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IMMUNOBIOLOGY OF A MURINE COCCIDIUM, *EIMERIA FALCIFORMIS* (APICOMPLEXA: EIMERIIDAE)

BY

© YUFANG SHI

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING, 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled IMMUNOBIOLOGY OF A MURINE COCCIDIUM, *EIMERIA FALCIFORMIS* (APICOMPLEXA: EIMERIIDAE), submitted by Yufang Shi in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The genetic and immunological interactions between a coccidium, *Eimeria falciformis*, and inbred stains of mice, *Mus musculus* were investigated. Genetic variation in immunity to infection was investigated by infecting three inbred and six congenic mouse strains with three successive oocyst inoculations. Variation in resistance was measured by oocyst production, delayed type hypersensitivity (DTH) reactivity and mortality. The results showed that genes of H-2 (mouse major histocompatibility complex) and also genes other than H-2 might share in controlling the immune response to eimerian infection, there was no close relationship among the measured criteria. There were significant differences in death rate and DTH reaction, but there was no significant difference in oocyst production among the three age groups.

Oocyst antigen specific DTH to eimerian infection was characterized. The hyperimmune mice exhibited a typical biphasic DTH response to the oocyst antigen. Adoptive transfer experiments showed that L3T4$^+$ T cells were required for the DTH reactivity. A T cell enriched spleen cell population from acutely infected mice suppressed the adoptive DTH, indicating the existence of infection-induced regulatory cells.

In some adoptive transfer experiments there were significant reductions in oocyst production by immune lymphocyte recipients. Nevertheless the DTH response was observed in all experimental animals.

Immune lymphocytes were stimulated *in vitro* with different parasitic antigens. The proliferation rates were significantly correlated with the concentration of cells and antigens. Anti-Thy-1 antibody reduced the proliferation rate of immune spleen cells to oocyst antigen.

The response of different subclasses of mouse serum antibodies to *E. falciformis*

infection were quantitatively compared in three inbred strains of mice with standard mouse immunoglobulin by using enzyme linked immunosubstrate assay (ELISA). Different strains had a variation in response in IgA and subclasses of IgG antibodies. The IgG response was greatly enhanced within three days after the second infection. It reached a peak at day 10 after the second infection and remained high for one month. Using an enzyme linked immunospot assay more IgG secreting cells were present in spleens than in mesenteric lymph nodes.

These observations are discussed in terms of the dynamics of the cellular and humoral immune response to this parasite.

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I dedicate this thesis to the Sino-Canadian friendship.

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIFT	Antigen induced footpad thickness
a	Type I error
ANOVA	Analysis of variance
B cell	Bone marrow derived lymphocyte
C'	Complement
CMI	Cell mediated immunity
Con-A	Concanavalin A
CPM	Count per minute
DCF	Direct centrifugal flotation
df	Degree of freedom
DPI	Days post infection
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
F	Variance ratio
H-2	Mouse major histocompatibility complex
HBSS	Hank's balanced salt solution
Ig	Immunoglobulin
ISC	Immune spleen cells
L3T4, Ly-2	T cell subpopulation specific surface proteins
MHC	Major histocompatibility complex

MLN	Mesenteric lymph node
OA	Oocyst antigen
P	Probability
PBS	Phosphate buffered saline
RC	Rabbit complement
SE	Standard error of the mean
SS	Sum of squares
T cell	Thymus derived lymphocyte
T_{DTH} Cell	Delayed type hypersensitivity mediating T cell
TDLs	Thoracic duct lymphocytes
T_H1, T_H2 Cells	T helper cell subsets
Thy-1	Mouse T cell specific surface marker
T.O.P.	Total oocyst production

I. INTRODUCTION

A. Coccidia

The coevolution of parasites and their hosts has resulted in precise ecological, genetic, morphological, physiological and immunological interactions. In this thesis the genetic and immunological interactions between a coccidium *Eimeria falciformis*, and mice *Mus musculus* are reported.

The coccidia are intracellular protozoan parasites which belong to the phylum *Apicomplexa*. The suborder *Eimeriorina* has 10 families, 37 genera and about 1,500 named species (Levine, 1982). The genus *Eimeria* in the family *Eimeriidae* is the largest genus containing more than 1,000 named species, occurring in invertebrates but mostly in vertebrates (Levine, 1982). They are markedly host specific, and are restricted to closely related species or subspecies (Joyner, 1982). There is also marked tissue specificity. Most species of *Eimeria* develop in epithelial cells of the intestinal mucosa with a remarkable degree of site restriction within the mucosa. Most host species are usually parasitized by several species of *Eimeria* [*e.g.* 11 species in chicken, *Gallus gallus*, and 15 in cattle, *Bos taurus* (Levine, 1982)].

The generalized life cycle of coccidia (Fig. I.1) consists of a specific number of successive cycles (varies with different species) of asexual reproduction (merogony) followed by the formation of gametes (gametogony). The fusion of a microgamete and a macrogametocyte forms the zygote which then develops a thick wall and is shed in the feces as a resistant cyst (oocyst). If environmental conditions are suitable, exogenous development (sporogony) follows during which sporozoites are formed. Oocysts are infectious only after sporulation is complete. When sporulated oocysts are ingested by a suitable host, excystation occurs and sporozoites penetrate the gut epithelial cells, thus

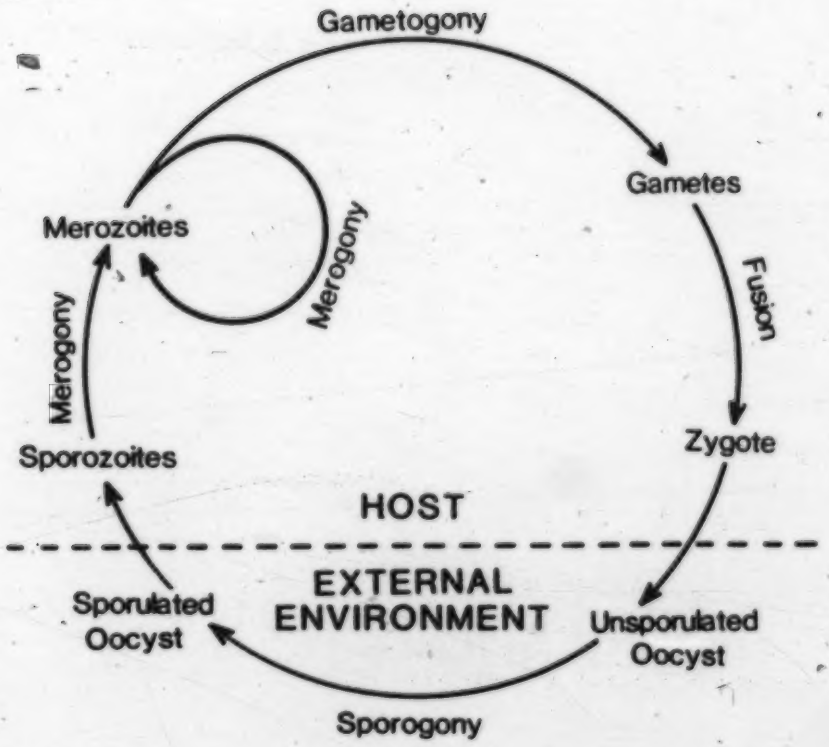


Fig. I.1. Schematic reprensentation of the basic life cycle of the order *Eucoccidiorida* (modified from Levine, 1982).

initiating merogony. Depending upon the pathogenicity of coccidian species, the parasitized gut cells show a range of effects varying from minimal interference with absorption of nutrients to death of infected cells. The infection progresses rapidly, and is self-limiting due to the fixed number of asexual generations (Rose, 1985).

In my experiments *Eimeria falciformis* was used. It is the type species of genus *Eimeria* having been first named by Eimer in 1870 (Owen, 1975). It is widely distributed in wild mice, and is one of the 15 eimerian species described from *Mus musculus* (Pellerdy, 1974). It is a pathogenic and immunogenic species which serves well as a model system. *Eimeria falciformis* has a prepatent period (before oocysts can be detected in feces) of seven days followed by a patent period of 10-16 days. After ingestion and excystation, the released sporozoites penetrate the mucosal epithelial cells in cecum and colon, and occasionally in lower ileum. This species has four morphologically distinct asexual generations. Mature first generation meronts form by 48 hour (hr), the second generation at 78 hr, the third at 114 hr, and the fourth at 144 hr post infection (PI). Mature macrogametocytes and microgametes appear at 156 hr PI (Mesfin and Bellamy, 1978).

B. Coccidiosis and Control

Pathogenic coccidian species may cause coccidiosis which is the pathological manifestation of this host-parasite interaction. Coccidiosis causes great economic loss in the poultry and livestock industry. Fayer and Reid (1982) reported that in 1980 in the United States alone 70 million dollars were spent on drugs for controlling chicken coccidiosis. The economic impact of coccidian infection world wide must be 100's of millions of dollars per year. Currently the most practical approach for controlling coccidiosis is chemoprophylaxis with limited use of immunological control.

Chemotherapy has been used for the treatment of coccidiosis since the development of sulphonamides in the early 1940's (Ryley, 1980). Many drugs have been developed for the anticoccidial market. The practice especially in the poultry industry of continuous low level medication to prevent outbreaks of coccidiosis has led to resistant strains of coccidia. With a few exceptions, most anticoccidial drugs introduced since 1961 have had a short market life span (Fayer and Reid, 1982). Furthermore, there are species and strain variations in sensitivity to anticoccidial drugs. The effort and cost of developing new drugs which meet government regulations have become so great that the pharmaceutical industry is becoming less and less interested in anticoccidial drug research (Ryley, 1980).

Alternative methods of coccidiosis control have had varied success. These methods include vaccination, host genetic resistance selection, special nutrient supplement, and sanitation management. Vaccination is becoming an attractive alternative to prophylactic medication. There are currently two approaches to this problem: 1) controlled exposure to virulent species of coccidia and 2) the development of attenuated strains of coccidian parasites for use as a vaccine. In the first approach a mixture of normal virulent oocysts from eight different species, is marketed as "Coccivac" for the chicken industry in the United States, and some other parts in the world. This "vaccine" is administered to young birds between 4-14 weeks old in the food or drinking water to initiate a mild infection to induce immunity. This method has been partially successful, but the negative factor is the potential for seeding of the environment with pathogenic species which may infect the next flock of birds. It may also fail to protect individuals that ingest too few oocysts, while those individuals who ingest too many may die. This method is not really vaccination, but rather mild infection with a low dose of several pathogenic coccidian species which stimulates protective immunity.

An alternative approach to immunization has been to attenuate virulent strains, either by repeated passage through embryos, by selection for nonpathologic strains from a pathologic species, or by irradiating virulent parasites. This method of vaccination has the technical problems of producing large numbers of oocysts, and sometimes poorly attenuated parasites may retain their pathogenicity (Rose, 1973). The *Escherichia coli* produced protein antigen may be a good vaccine, but needs extensive field trials (Clark et al., 1986). This method has provided good protection against challenge with viable oocysts. The vaccine is administered by muscular injection. The protective mechanism of this protein is not understood, and its potential for widespread application is still unknown. Immunization appears to be a most promising way to control coccidiosis. The best approach to immunological control of coccidiosis may be the use of genetic engineering to identify, and then produce a functionally defined parasitic antigen(s) as a vaccine.

C. Immunological Aspects of the Host-parasite Interaction

Investigation of the mechanism of immunity to coccidian parasites is an active area of research, but the mechanism(s) by which coccidian parasites stimulate or evade host immunity is far from clear. We need a clearer view about which parasite antigens are involved in stimulating host immunity, which partition of the immune system recognizes the functional antigen(s), how the host immune system is regulated during infection, and the role of genetics in controlling host susceptibility.

1. Genetically Controlled Host Susceptibility

The intracellular coccidian parasites are not only highly host specific, they may also be strain specific. Klesius and Hinds (1979) infected eight strains of mice with *E.*

ferrisi. Susceptibility varied with different doses of inoculated oocysts in different strains. They crossed sensitive C57BL/6 to resistant strains, CBA, C3H/Anf and DAB/2, and found that F1's were resistant. Thus, the resistance was characterized as a dominant genetic expression. Some F1 hybrids were more resistant than either of their parent strains. This indicated that the resistance may be multiple-gene controlled, or there may be a gene complementation in the resistance gene region(s). They suggested that the gene location for resistance to coccidiosis could be best determined with congenic strains of mice. Mayberry et al. (1982) infected different strains of mice with a rat *Eimeria*, and found that mouse susceptibility to the rat *Eimeria* was influenced by murine host genotypes.

Rose et al. (1984) studied the susceptibility to *E. vermiformis*, another mouse coccidium, in seven recombinant strains of BALB/c X C57BL/6, and found that the resistance/susceptibility to this parasite was not associated with the H-2 locus. Unfortunately the use of those recombinant strains was unsatisfactory for H-2 study because the strains were different not only at H-2 locus, but also at many other gene regions (Klein, 1975). This makes it is difficult to determine the function of H-2 genes. The relationship between the H-2 locus and the immune response to coccidian infection still remains to be determined by using congenic strains. Rose et al. (1985) used whole body irradiation and injection of cortisone acetate or cyclophosphamide in resistant BALB/c and susceptible C57BL/6. They found that oocyst production was based on the immune response. This suggested that host resistance to *E. vermiformis* was determined by some host genes. Stockdale et al. (1985) infected five inbred strains of mice with *E. falciformis*. Although there were differences in susceptibility, all the strains of the mice were highly resistant to reinfection. Unfortunately they only measured the oocyst production which may not be the best assay of the immune response (see discussion

section). Rose and Millard (1984) studied the infectivity of *E. vermiformis* in immunologically impaired rats. The parasite showed some degree of normal life cycle development in abnormal tissue and abnormal hosts. Again this was evidence that host immunity to eimerian infection was controlled by host genetic background.

The immune response varies among the different host genotypes (Lillehoj and Ruff, 1987). The relationship between resistance to coccidian infection and the major histocompatibility complex (MHC) still remain poorly understood (Rose et al., 1984).

2. Acquired Humoral Immunity

Circulating antibodies to *Eimeria* have been demonstrated in many host species and detected with several kinds of immunological techniques (Rose, 1982). Different developmental stages and different species have both common and specific antibodies. Rose (1977) demonstrated an Arthus-type response. Antibodies can be detected within one week after infection. Rose and Mockett (1983) detected IgG in chicken serum and egg yolk after infection with different eimerian species at different endogenous stages using an enzyme linked immunosorbent assay (ELISA). Lillehoj and Ruff (1987) examined the subclass specific antibody response in two inbred strains of chicken, and found no significant difference in antibody production in the two strains. The most important antibody was thought to be IgA. Secreted IgA in the intestinal lumen can reduce the ability of the sporozoites to penetrate and to differentiate (Davis and Porter, 1979; Rose and Hesketh, 1987). Opsonic and cyclophilic antibodies can enhance the uptake of parasite by macrophages. Rose and Hesketh (1979) used bursectomised chicken and found the resistance to the secondary infection of *E. tenella* was slightly lower than that in normal chickens. Overall, antibodies to coccidian infection occur, but may only play a minor role in the immune response (Rose and Hesketh, 1979).

Like other intracellular parasites immunity to coccidia depends on the proper functioning of T lymphocytes. T cells may be necessary for the stimulation of B cells to produce antibodies to *Eimeria*, and the antibodies maybe involved to some extent in protective immunity. Antibodies may be directed against the parasitic stages when they are extracellular. However, most of the immune response is mediated by T lymphocytes (Rose, 1982), probably through delayed type hypersensitivity, or other lymphokine-induced phenomena

3. Acquired Cellular Immunity

The knowledge of cell mediated immunity (CMI) to *Eimeria* is steadily expanding along with the understanding of the function of T-lymphocytes and the development of new techniques. Adoptive transfer of large numbers of immune lymphocytes to syngeneic animals greatly reduced infection with *Elmeria* species in recipients, such as in rats (Liburd et al., 1973; Ogilive and Rose, 1977) and chickens (Rose and Hesketh, 1982). However, in those transfers lymphocytes were not fractionated and consisted of cells involved in both humoral and CMI response. Therefore, it is still unknown which type of cells are responsible for adoptive transfer of immunity. Rose and Hesketh (1982) found intravenous injection of spleen cells or peripheral blood lymphocytes in chickens caused a significant reduction of oocyst production in challenged recipients, but again the immunocytes were not fractionated. The effective lymphocyte subset(s) has not been demonstrated. Mesfin and Bellamy (1979) and Rose and Hesketh (1979) used athymic animals to determine the role of T lymphocytes. Primary infections in those animals were enhanced. Athymic animals were completely susceptible to reinfection; thus they concluded that the immunity was largely or wholly, thymus dependent. It is important to remember that the deficiency of all T

cells (including T helper cells) in athymic animals affects both the humoral and CMI. A lymphokine obtained from mitogen-stimulated bovine T cells induced cultured bovine monocytes to inhibit intracellular development of the first generation of meronts of *E. bovis* and *E. papillata* (Speer et al., 1985); this could be considered as a direct evidence to show the function of T cells. Liburd et al. (1972) dialyzed transfer factor from lymph nodes of immunized rats, and injected this factor into normal rats causing a significant reduction in oocyst production. However, the existence of transfer factor is still an open question, and the term "transfer factor" is becoming less used in the literature. Long and Miline (1971) found fewer oocysts were excreted by chickens treated with an inducer of interferon, and Fayer and Baron (1971) claimed that interferon inhibited the *in vitro* development of *E. tenella*.

The delayed type hypersensitivity (DTH) response to many intracellular pathogens has been well documented. It is a T cell mediated immune phenomenon characterized by an inflammatory reaction at the site of antigen challenge. The DTH response to coccidian parasites was first reported in man and guinea pig infected with *Toxoplasma gondii* (Frenkel, 1948). This has been a useful diagnostic technique for human toxoplasmosis. DTH in eimerian infection has been investigated in several domestic animal species. Klesius et al. (1976) studied the DTH response in rabbits infected with *E. stiedai* and found a long lasting positive reaction. Klesius et al. (1977) also studied *E. bovis* in cattle and found the DTH response was comparable to the tuberculin DTH reaction. DTH also occurs in avian coccidiosis (Rose, 1977). Giambrone et al. (1980) found a positive correlation between resistance and the DTH reaction in chicken coccidiosis.

Different T cell subsets have different roles in the immunity to parasitic infections. The information on the interaction between parasite and T cell subsets is essential for

vaccine investigation and also tell us about what happens at the host-parasite interface (Mogil, 1985). Lillehoj (1985) used flow cytometric analysis and found L3T4 and Ly-2 receptor bearing T cells increased in the lymphoid system after coccidian infection. IL-2 receptor bearing T cells did not appear in the first infection, but increased in the second infection. Lillehoj (1987) studied the effect of cyclosporin A and hormonal bursectomy in *E. falciformis* infection, and concluded that the CMI was the major role in host protective immunity. Bhogal et al. (1986) studied T lymphocyte clones with specificity for *E. tenella* by using *in vitro* antigen stimulation and the cooperation of T cells, B cells, and antigen presenting cells. The role of T cell subsets in the development of the immunity to some other parasites, such as *Plasmodium* (Mogil, 1985; Jayawardena et al., 1982; Gross et al., 1984; Greavely and Kreier, 1976), *Leishmania* (Liew, 1982; Poulter, 1979 and 1980; Poulter and Pearce, 1980) and ticks (Wikel, 1984) have been extensively studied in the recent years. The same approach can be applied to the study of cell mediated immunity to *Eimeria*.

D. Thesis Focus

The objectives of this thesis were to study the immunological response of hosts to parasitic infection by using *E. falciformis* and *Mus musculus* as a model system. Mice infected with this parasite develop strong resistance (Stockdale et al., 1985), and the life cycle of this coccidian species is well known and predictable (Mesfin and Bellamy, 1978). The role of host genetics and resistance is poorly defined. The DTH response also needs clarification. Coccidian infection induces humoral and cellular immune responses (Rose, 1982), however the functional arm is still unanswered. The major focus of my investigations were:

1. Host genetics and susceptibility to *E. falciformis* infection.
2. The characterization of delayed type hypersensitivity to murine coccidian

infection.

3. Lymphocyte subsets involved in the immunity to coccidian infection.

4. The antibody response of infected animals, and the relationship of CMI and antibody response to infection.

II. MATERIALS AND METHODS

A. General Materials and Methods

1. Animals

Commercially available inbred and congenic strains of mice were used in all experiments. They were all male mice and had at least one week of acclimatization following delivery before experimental use. Feces were checked for the presence of helminth eggs or coccidian oocysts by using a Sheather's sugar solution (500 g sucrose, 6.5 g phenol in 320 ml water) in a direct centrifugal flotation (DCF) method (Levine, 1973). Mice were individually caged in a negative air isolating hood after infection to prevent spreading of infectious oocysts. Feed and water were provided *ad libitum*. Mice were age-matched within each experiment.

2. Parasite

Oocysts of *Eimeria falciformis* were originally obtained from Dr. C.A. Speer, Veterinary Research Station, Montana State University, Bozeman, MT, U.S.A.. Oocysts were first passed through C57BL/10 mice to increase oocyst numbers for further experimental use. Oocysts were separated from feces by first sieving with a serial meshes and then using flotation and glass petri dish adhesion methods with Sheather's sugar solution. The oocysts were then sporulated in aerated aqueous 2.5% $K_2Cr_2O_7$ for three to four days and stored in 2.5% $K_2Cr_2O_7$ at 5 ^{o}C for less than two months before use. Oocysts were washed three times with distilled water before being used for infecting mice.

3. Infection

Mice were anaesthestized with CO_2; oocysts were then inoculated with 0.3 ml distilled water by using a 1 cc syringe connected to a stomach tube. Since different

dosages were used in some experiments, they will be specified in their respective results section.

4. Antigen Preparation and DTH Testing.

Oocyst antigen (OA) for DTH testing was prepared at the concentration of 10^6 sporulated oocysts/ml in sterile phosphate buffered saline (PBS, pH 7.2) or Hank's balanced salt solution (HBSS) (Sigma Chemical Co, St. Louis, MO., U.S.A.). Oocysts were ruptured in a French Pressure cell at 15,000 pig (Klesius et al., 1976) or by sonication, and were microscopically checked for oocyst breakage. The prepared OA was stored at - 20°C.

Soluble OA for the enzyme linked immunosorbent assay (ELISA), and for the enzyme linked immunospot (ELISPOT) assay was prepared with the same methods as stated for the DTH, except with a higher concentration of oocysts (10^7/ml). This oocyst suspension was then centrifuged and the soluble portion was collected for use as ELISA antigen. The optimal antigen protein concentration was 1.1 mg/ml as measured with a spectrophotometer at 595 nm.

For DTH testing OA at a concentration of 8,000 oocysts in 25*ul* PBS was injected into the right footpad. The left footpad was injected with 25*ul* PBS as the control. The Ag induced footpad thickness (AIFT) was derived from the following formula:

$$(R_{xhr}-L_{xhr})-(R_{0hr}-L_{0hr})$$

(R for right footpad; L for left footpad; x for time DTH was measured).

5. Assessment of Oocyst Production

Fecal collection began on day 7 postinfection (DPI), and continued at two-day intervals. Feces were collected from individually caged mice into aluminum cake pans containing a solution of 2.5% $K_2Cr_2O_7$ which inhibited bacterial growth and dehydration of feces. Collections were continued until the end of oocyst excretion (15-16 DPI). Oocysts produced on the last two days by dying mice were excluded from the assay. The

oocysts were counted in McMaster chambers using Sheather's sugar solution for flotation (Long et al., 1976). Toward the end of the patent period oocysts were counted with the DCF technique which was more sensitive at low numbers.

6. Statistics

Programs in SAS (SAS Institute Inc., 1982) and BIOM (Rohlf, 1985) were used for data analysis. Specific tests are indicated in respective sections.

B. Murine Major Histocompatibility Complex and the Immune Response to *Eimeria falciformis*

1. Animals

Three inbred strains (BALB/c, C57BL/10 and C3H/He) of three age groups (28, 42 and 108 days old) and six adult (70 days old) congenic strains (B10.PL, B10.BR, B10.D2, C57BL/10, B10.A and B10.M) were purchased from Jackson Laboratory, Bar Harbor, ME, U.S.A. Each group had 10 mice, and were age-matched.

2. Infection

In the first experiment with three inbred strains each mouse received three inoculations; 500 sporulated oocysts on day 0; 1000 on day 2 and 1500 on day 4. Each mouse was inoculated with 500, 800, and 800 oocysts on days 0, 2, and 4, respectively. A multiple infection schedule was followed since Joyner and Norton (1974) showed that small repeated dosages of coccidia were more immunogenic than a single inoculation. Clare et al. (1985) also used an immunization schedule of 500 oocysts of *E. tenella* daily for 5 days in chickens, and showed the effects of chicken MHC on acquired immunity to coccidiosis. The MHC was previously thought to have no effect in mice (Rose et al., 1984). After primary infection all mice were challenged on 30-32 DPI with 20,000 oocysts; oocyst numbers were counted as stated previously.

C. Characterization of Murine Delayed Type Hypersensitivity Response to Coccidian Infection and Adoptive Lymphocyte Transfer

1. Animals

Male C57BL/6 mice were purchased from Charles River Laboratory, Quebec, Canada. Each mouse received a primary infection of 500 oocysts followed 30 days later with a challenge infection of 20,000 oocysts. These hyperimmune mice were then used as donor mice. The recipient mice were 42 days old at cell transfer. In some experiments 28 day-old mice were used, while in other experiments the recipient mice were irradiated before cell transfer (details included in results section).

2. Preparation of Cells for Adoptive Cell Transfer.

Spleens from immune mice were used as the cell source for adoptive cell transfer. Single cell suspensions were prepared by grinding spleen pieces between frosted ends of microscope slides. Cells used for immunosuppression experiments were harvested from spleens of mice 10 DPI which each received a primary infection of 500 sporulated oocysts.

Thy-1 negative spleen cells were prepared by treating whole spleen cell suspensions at the concentration of $1X10^7$ cells/ml with monoclonal anti-Thy-1,2 (New England Nuclear) in HBSS at room temperature for 45 minutes. The anti-Thy-1 treated cell suspension was then centrifuged at 250 g for 10 minutes. The pelleted cells were then resuspended in rabbit complement (1:10 with HBSS) to approximately $1X10^7$ cells/ml. The suspension was then subjected to incubation at 37°C for 30 minutes. Ig negative (T cell enriched) cell populations were obtained with goat anti-mouse Ig conjugated mouse Ig coated glass bead columns (SCI-CAN Diagnostics, Edmonton, Alberta, Canada) according to the method described by Wigzell (1976). Monoclonal anti-L3T4 (GK1.5) (ATCC, clone) was used to deplete L3T4 cells from T cell enriched spleen

cells as described above. Cells were assessed for viability by trypan blue stain uptake. Live cells were counted with a hemacytometer. Each recipient received an i.v. injection of $3X10^7$ unfractionated spleen cells, or the corresponding complement of Thy-1$^-$, Ig$^-$, or L3T4$^-$ spleen cells.

D. *In vitro* cell culture

In the first experiment spleen cells were harvested from a 70 day old mouse which had been infected with 2,000 sporulated oocysts 30 days earlier. These cells were cultured in 50ul RPMI-1640 (10% fetal bovine serum, 2mM L-glutamine, 0.015M Hepes and antibiotics were added) with whole oocyst antigen in microtiter plates at 37°C in 7% CO_2 in humidified atmosphere for 48 hr, and then pulsed with radiolabelled thymidine. After 24 hr the cultured cells were collected and proliferation was assayed by measuring the amount of newly synthesized DNA (Butcher and Ford, 1986).

In the second experiment three kinds of antigens were used: 1) whole oocyst antigen, 2) oocyst walls-centrifuged particulate antigen , 3) merozoite antigen [sonicated purified second generation of merozoites (Speer et al.,1985; Klesius et al., 1976)]. Each antigen had two concentrations of oocysts or merozoites, 5,000/well and 10,000/well. Lymphocyte suspensions were also at two concentrations, $2.5X10^5$/well and $5X10^5$/well. All of the other methods were the same as in *in vitro* culture experiment 1.

In the third experiment spleen cells were harvested from mice which had been infected twice; first with 1,000 oocysts and later challenged on day 30 with 20,000 oocysts. One-half of the spleen cells was treated with anti-Thy-1 monoclonal antibody and rabbit complement, while the other half was treated with rabbit complement only. These two fractions were cultured at two concentrations ($2.5X10^5$/well, $5X10^5$/well), and with oocyst antigen (10^3/well). All of the other methods were the same as described previously in experiment 1.

E. Antibody Response in *Eimeria falciformis* Infection

1. Serum

Blood was collected from the tail vein (heated by infrared light) into microtainer tubes (Becton Dickison and Company, NJ, U.S.A.). Collected blood was allowed to clot for a minimum of 30 minutes, and then centrifuged for 2-3 minutes at 11,000 g. Serum was stored at -80°C.

2. Reagents and solutions

PBS(pH 7.2) was used throughout the experiment. The washing solution was made of PBS with 0.05% Tween 20. The blocking solution was 1% gelatin in PBS. Diluent for serum was 0.5% gelatin in PBS. MonoAb-ID EIA Kit and peroxydase-Goat anti-mouse IgG (H+L) were purchased from Zymed Laboratories, San Francisco, CA., U.S.A.. Substrate was prepared fresh each time. It was made up with the following formula: 5ml critic buffer, 50 ul 2% ABTS (2,2-azino-di[3-ethyl benzthiazoline sulfonic acid]) in double distilled water and 20 ul 0.5% H_2O_2. Standard IgG subclasses and IgA were obtained from Sigma Chemical Co, St. Louis, MO., U.S.A.

3. Procedures

Soluble antigen was diluted with PBS to 2 ug/ml. Fifty microliters of the diluted antigen was added into each well of flat bottomed Nunc-Immuno plates (Inter Med, Denmark) to allow antigen protein to attach to the well surface. The coating plates were incubated at 4°C for at least 24 hr, and then washed three times with washing solution. The antigen coated plates were treated with 1% gelatin for 2 hr at 37°C to block the remaining nonspecific binding sites. The test serum was successively diluted two fold in diluent with a starting dilution of 1:20 or 1:200 depending on which subclass of antibody was being tested, and when the serum was collected. After one hour incubation at 37°C the plates were washed four times with washing solution. Fifty microliters of 10 times diluted kit rabbit anti-mouse specific heavy chain for inbred strain comparison test, or

1:4000 diluted peroxidase conjugated goat anti-mouse IgG(H+L), were added and the plates were incubated for 1 hr at 37°C. For the comparison test the plates were washed four times with washing solution, and then incubated with 50 ul diluted (kit formula) peroxidase conjugated goat anti-rabbit (IgG+M+A) for 1 hr at 37°C. The plate was then washed with washing solution at least five times. The enzyme activity was indicated by adding freshly prepared substrate solution. The enzyme catalyst ability appeared as the density of colour. The absorbance was read at 405 nm with a ELISA reader.

For standard curves, standard immunoglobulin at a concentration of 0.05 mg/ml in PBS were initially used, and then a two fold dilution series was carried out across two plates. The remaining space was then blocked with blocking solution. All the other procedures were the same as described above. Each group had 4-10 samples. The absorbances were averaged and plotted. The antibody concentration in the serum was measured with the standard curve.

The quantitative antibody concentrations in serum were determined by selecting the optimal standard concentration of immunoglobulin which gave a straight slope including at least four points. Then the concentrations were plotted against the absorbance (405 nm). The correlations which had a correlation coefficient greater than 0.95 ($R>0.95$) were used. A value from the serum curve which also rested on a straight portion of the plotted slope was selected for antibody concentration determination. The best value was those absorbance between 0.050 to 0.200 (personal communication, Dr. Stemke, Department of Microbiology, University of Alberta). The serum antibody values were obtained by multiplying the calculated value with the dilution factor.

F. Enzyme-linked immunospot assay for antibody secreting cells

The enzyme linked immunospot assay (ELISPOT) method was modified from

Czerkinsky et al. (1987). Briefly, immune spleen cells and mesenteric lymph node cells were collected from C57BL/6 mice nine days after the third challenge infection. Non-immune spleen cells from specific pathogen free C57BL/6 mice were controls. Plastic petri dishes (35 mm in diameter) were coated with 0.05 mg soluble oocyst antigen in 1.5 ml PBS at 4°C for 24 hr. The antigen coated dishes were then incubated with 2% gelatin at 37°C for 2 hr to block the remaining binding sites on the plastic dishes. Cells were prepared and counted as previously stated. Different concentrations of cells were cultured in 300 ul (to avoid the spread of antibody, but with just enough media to cover the the plastic surface) medium (same as in section D) to each Ag coated dish. The cells were then cultured at 37°C in a 7% CO_2 humidified atmosphere on a leveled surface for 3-4 hr. During the culture period any disturbance should be avoided. The cells were discarded and rinsed with PBS 2 times. In order to elute the remaining cells, the petri dishes were incubated with 3 ml of 0.05 M EDTA in PBS for 15 minutes, and then washed with PBS tween 20 three times and kept dishes covered to prevent drying. To each dish 2.5 ml 1:300 peroxydase conjugated goat anti-mouse IgG (H+L) in PBS tween was added and then incubated at 37°C for 1 hr. The dishes were then washed with PBS tween 4 times. Three to 5 ml of gel-substrate (50 mg p-phenylenediamine dissolved in 2 ml methanol and 50 ul 30% H_2O_2 added into 100 ml 1% Noble agar) was added to each dish and the excess agar was removed by shaking it off (to get a good gel membrane the gel-substrate must be kept at 45-48°C in a water bath). The dishes were allowed to gel for two minutes. Spots began to appear in 3-5 min. The number of spots were counted 30 min later (the spots are very stable) with the low magnification of a stereomicroscope.

III. GENETIC CONTROL OF MURINE IMMUNE RESPONSE TO *EIMERIA FALCIFORMIS*

Two experiments were conducted to study genetic interaction of host and parasite. In the first experiment the differences of susceptibility in three age groups of three inbred strains of mice to infection with *Eimeria falciformis* were studied. The second experiment investigated the effects of the major histocompatibility complex (MHC, H-2 in mouse) on the susceptibility to eimerian infection, and the effects of non-H-2 genes on susceptibility were assessed by combining the results from both experiments. The variations of host resistance were measured by oocyst production, delayed-type hypersensitivity (DTH) reactivity, and mortality. There were significant differences in the above criteria among the three inbred strains and also the six congenic strains. These results indicate that the genes of the H-2 complex and non-H-2 genes may share in controlling the immune response to eimerian infection. There was no close relationship between oocyst production and the immune response in terms of specific DTH reaction level. There were significant differences in death rate and DTH reaction, but there was no significant difference in oocyst production among the 3 age groups of the 3 inbred strains. C57BL mice were selected as the suitable mouse strain for further experiments.

Comparison of Oocyst Production. In Experiment 1 three inbred strains (BALB/c, C57BL/10 and C3H/He) of three age groups (28, 42 and 108 days old) were used. The data are summarized in Table III.1. Oocyst production was analyzed with 3-way ANOVA by using strain, age and fecal collection date as the three factors (Table III.2). The oocyst production in different inbred strains was significantly different ($P<0.001$). The data also confirms that C57BL/10 was "susceptible" strain in terms of oocyst

Table. III.1. Mean total oocyst production and death rate of three inbred strains and three ages of mice infected with *Eimeria falciformis*.

Mouse Strain	H-2 Haplotype	Age (days)	N[a]	Death Rate (%)	DTH Level[b]	Mean total oocysts ($X10^6$, 95%C.L.[c])
BALB/c	d	28	10	60	24.3±2.6	17.6(14.72-20.51)
		42	10	10	26.2±1.3	17.0(15.01-18.93)
		108	10	0	28.8±1.8	14.7(13.65-15.79)
C57BL/10	b	28	9	78	--------	33.6±3.22(SE)
		42	9	56	30.0±4.0	27.7(17.37-38.10)
		108	10	0	32.8±1.3	28.7(27.03-30.40)
C3H/He	k	28	10	100	----------	----------
		42	10	80	----------	11.5±0.10(SE)
		108	10	40	23.4±1.6	7.2(5.44±8.93)

a: number of mice infected.

b: Oocyst antigen induced footpad thickness, 24 hr measurement (Mean±SE).

c: C.L. = 95% confidence limits used only with sample size of 4 or more.

production (Stockdale et al., 1985). There were no differences between age groups in oocyst production ($P>0.05$). Oocyst collection days had a significant influence ($P<0.001$) due to the specific life cycle of the parasite; this also lead to a significant interaction between strain and date ($P<0.001$). The overall oocyst production of each strain was significantly different from each other as showed by GT2-test (all P's<0.05). This indicates that the host genetic background controlled the level of proliferation of this coccidian parasite. Because there was no difference between age groups, only the oocyst production for the 108 day-old mice of each strain is presented in Fig. III.1.

In Experiment 2 six congenic strains (B10.PL, B10.BR, C57BL/10, B10.A, B10.D2 and B10.M) were used. Oocyst production of these strains was analyzed with a two-way ANOVA with strain and fecal collection date as the two factors (Table III.3). Oocyst production in six congenic strains was significantly different ($P<0.001$) (Fig. III.2 and Table III.4). GT2-test showed that some strains had different levels of oocyst production (Table III.5). This indicated that the H-2 genes in different strains had different levels of control over oocyst production.

There was no difference in oocyst production in B10.BR versus B10.D2 and B10.A versus B10.BR, while there was a significant difference in B10.A versus B10.D2. This could not be readily explained. This suggests that oocyst production was controlled by multiple genes (Mahrt and Shi, 1988). Genes other than H-2 also share in the control of susceptibility to *E. falciformis* infection. Analysis of oocyst production (two way ANOVA, Table III.6) from paired strains of the same haplotype, but with different genetic background [BALB/c and B10.D2 (H-2 haplotype d), and C3H/He and B10.BR (H-2 haplotype k)] was significantly different ($P<0.05$ for $H\text{-}2^d$, $P<0.001$ for $H\text{-}2^k$). This shows that the non-H-2 genes also influence host susceptibility to this parasite. There was no difference ($P>0.05$) in oocyst production in C57BL/10 mice used in the two experiments

Table III.2. ANOVA table: Oocyst production of 3 inbred mouse strains with 3 age groups infected with *Eimeria falciformis*.

Source of variation	df	SS	F	P
Strain	2	427.52	94.47	0.0001
Age	2	10.00	2.21	0.1118
Date	4	1674.25	184.99	0.0001
Strain * Age	4	4.80	0.53	0.7135
Strain * Date	8	401.34	22.17	0.0001
Age * Date	8	22.11	1.22	0.2866
Strain * Age * Date	9	4.81	0.24	0.9889
Error	263	595.07		

Table III.3. ANOVA table: Oocyst production of six congenic mouse strains.

Source of variation	df	SS	F	P
Strain	5	306.04	11.74	0.0001
Date	4	2515.01	120.63	0.0001
Strain*Date	20	449.11	4.31	0.0001
Error	220	1146.72		

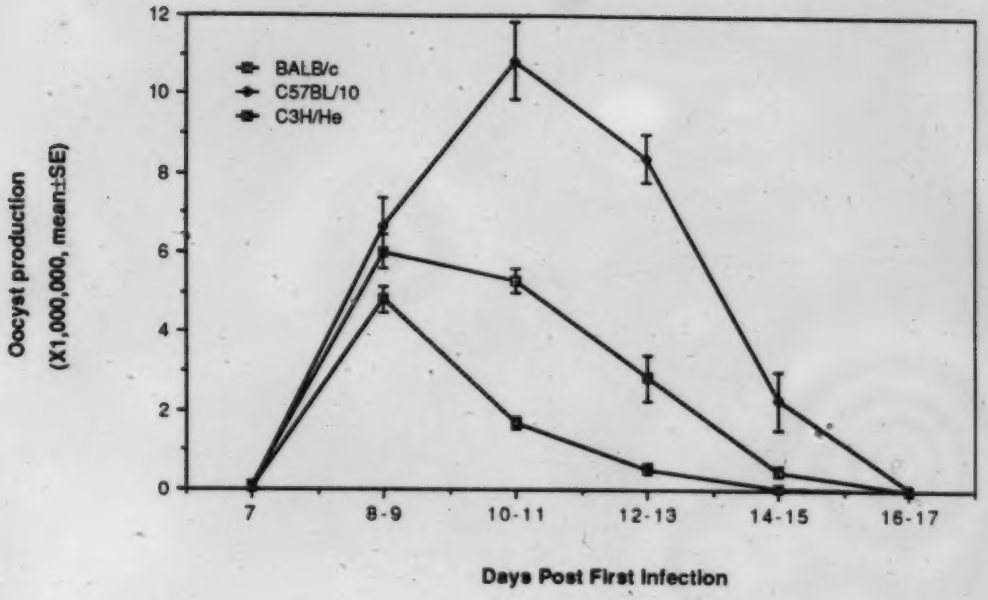


Fig.III.1. Oocyst production of adult groups (108 days old, 9-10 mice per group) of inbred strains C57BL/10, BALB/c and C3H/He after three successive infections (500 sporulated oocysts on day 0, 1000 on day 2 and 1500 on day 4) of *Eimeria falciformis*.

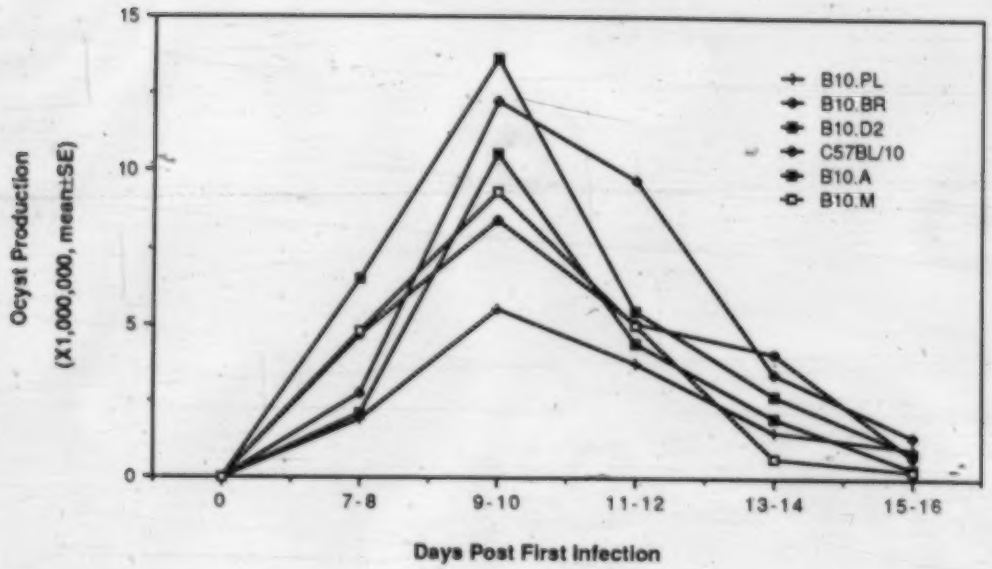


Fig.III.2. Oocyst production of 6 congenic mouse strains; C57BL/10, B10.A, B10.M, B10.D2, B10.BR and B10.PL infected with three successive infections (500, 800, and 800 sporulated oocysts on days 0, 2, and 4, respectively). Each group had 9-10 mice at the beginning of the experiment.

Table III.4. Mean total oocyst production, death rate and DTH level of six congenic strains of mice infected with *Eimeria falciformis* at 70 days old.

Mouse Strain	H-2 Haplotype K	IA_β	IA_α	IE_β	IE_α	D	N[a]	Death Rate(%)	DTH Level[b]	Mean Total Oocysts ($X10^6$) (95% C.L.[c])
B10.PL	u	u	u	u	u	u	9	22	76.2±9.7	13.6(10.17-17.02)
B10.BR	k	k	k	k	k	k	10	30	62.5±7.1	22.8(16.06-28.59)
B10.D2	d	d	d	d	d	d	10	20	73.2±5.8	19.3(16,82-21.67)
B10.A	k	k	k	k	k	d	10	0	107.7±5.3	29.2(23.65-34.76)
B10.M	f	f	f	f	f	f	10	0	62.0±7.9	20.0(16.10-23.87)
C57BL/10	b	b	b	b	b	b	10	0	28.8±4.7	29.9(26.60-33.28)

a. Number of mice at the start of the experiment.

b. Oocyst antigen induced footpad thickness, 24 hr measurement (X0.01 mm, Mean±SE).

c. 95% confidence limit.

Table III.5 Multiple comparison of oocyst production among six congenic mouse strains with GT2-test (α=0.05 indicated by '*').

	B10.A	B10.BR	B10.M	B10.D2	B10.PL	T.O.P.[$]
C57BL/10	---	---	*	*	*	29.9
B10.A		---	*	*	*	29.2
B10.BR			---	---	*	22.8
B10.M				---	---	20.0
B10.D2					---	19.3
B10.PL						13.6

$: Mean total oocyst production ($X\ 10^6$).

Table III.6. ANOVA tables: The effect of non-H-2 genes on oocyst production.

a). H-2b (2 C57BL/10 groups from two experiments).

Source of variation	df	SS	F	P
Strain	1	1.84	0.42	0.5170
Date	4	1263.67	72.61	0.0001
Date*Strain	4	340.91	19.59	0.0001
Error	90	391.58		

B. H-2d (B10.D2 and BALB/c).

Source of variation	df	SS	F	P
Strain	1	9.64	4.78	0.0317
Date	4	628.76	77.97	0.0001
Date*Strain	4	63.13	7.83	0.0001
Error	80	161.29		

c). H-2k (B10.BR and C3H/He).

Source of variation	df	SS	F	P
Strain	1	208.78	88.53	0.0001
Date	4	320.74	34.00	0.0001
Date*Strain	4	47.41	5.03	0.0015
Error	60	141.49		

(Fig. III.3 and III.6). This suggests that the oocyst production from the two experiments was comparable.

In the above experiments the life cycle in terms of daily oocyst output followed the same pattern (Fig. III.1 and III.2).

Comparison of Mortality. The mortality of the three inbred strains of three age groups of mice in experiment 1 infected with *E. falciformis* is shown in Fig. III.4. The cumulative mortality data were rank transformed (Covover and Iman, 1981), and then analyzed by a three-way ANOVA with strain, age and time after infection as the factors (Table III.7). The death rate was significant among strains ($P<0.01$), and also significant among age groups ($P<0.001$). Mice of C3H/He were the most susceptible at any age level, while the mice of BALB/c strain were the most resistant. The effect of host age on mortality from coccidiosis is controversial (Long, 1973). The data in this experiment clearly support the premise of young animals being more susceptible than older ones in the three inbred strains of mice tested.

The level of resistance to infection with *E. falciformis* was also examined in six congenic strains in experiment 2 (Table.III.4). The following two categories of resistance were apparent: resistant and susceptible. When the survival of 100% of the infected animals over the patent period was used as the criterion of a "resistant" strain, then strain B10.A, C57BL/10 and B10.M were characterized as resistant. In contrast, B10.BR, B10.D2 and B10.PL mice were susceptible. In the latter strains 20-30% died during the patent period. When mortality was used as a measure of susceptibility between different congenic strains, the H-2 genes clearly control the susceptibility of mice to coccidian infection.

An explanation for the mortality comparison of B10.D2 and B10. BR to B10.A is difficult. One possibility is that the H-2 genes were not the genes which

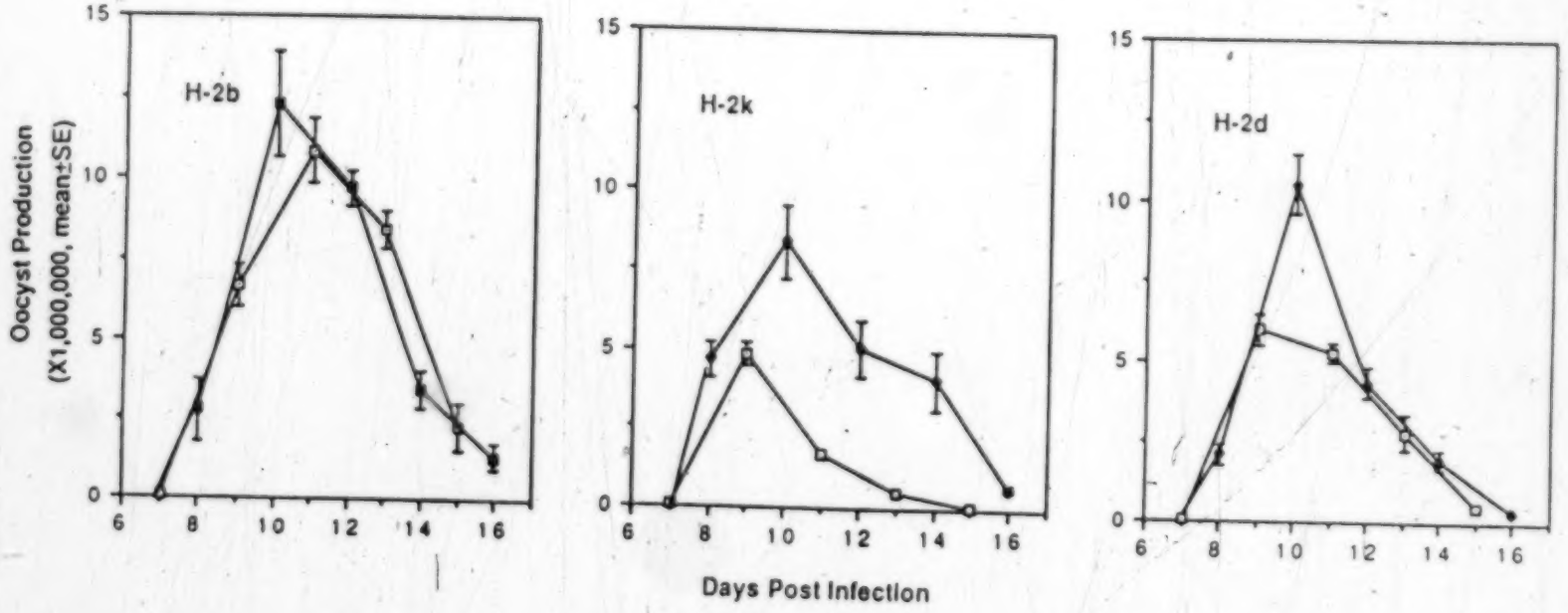


Fig. III.3. Comparison of oocyst production from the paired strains of mice which have the same H-2 haplotypes [BALB/c and B10.D2 (H-2 haplotype d), C3H/He and B10.BR (H-2 haplotype k), and two C57BL/10 (H-2 haplotype b)], infected with *Eimeria falciformis*; bars above and below symbols indicate standard error.

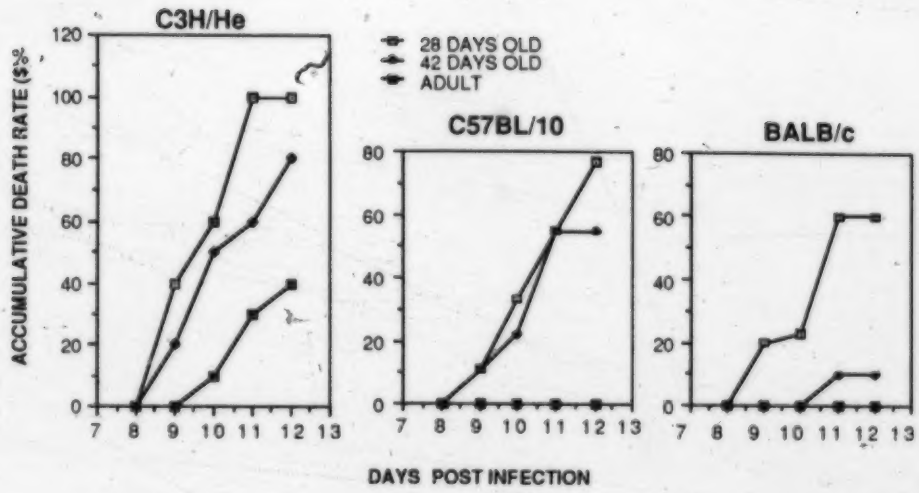


Fig.III.4. Cumulative death rate of three inbred mouse strains with three age groups infected with three successive infections (500 sporulated oocysts on day 0, 1000 on day 2 and 1500 on day 4) of *Eimeria falciformis*. Each group had 9-10 mice at the beginning of the experiment.

Table III.7. ANOVA table: Death rate in three inbred mouse strains with three age groups in each strain (data were rank transferred).

Source of Variation	df	SS	F	P
Age	2	13353.72	16.52	0.0001
Strain	2	6946.72	8.59	0.0013
Age*Strain	4	1455.78	0.90	0.4775
Error	27	10912.75		

influenced mortality, or there was an unusual class I and class II gene interaction which led to the difference of mortality.

The death rate of mice in Experiment 2 was compared to the adult groups of the three inbred strains in Experiment 1 for the following reasons: 1) all C57BL/10 in the two experiments survived; 2) the mice in both experiments were all adult because they were all over 60 days old. BALB/c and B10.D2 are all H-2^d haplotype. No BALB/c died, but 20% of the B10.D2 died. Also the death rate was different between C3H/He and B10.BR. Those data suggest that the non-H-2 genes also control the host susceptibility.

Comparison of DTH Response. Twenty-four DPI antigen specific footpad swelling response occurred following stimulation by nonviable antigen prepared from shattered oocysts. The swelling response was a typical DTH swelling pattern (illustrated in chapter IV). The DTH reaction in adult mice of the 3 inbred strains was significantly different ($P<0.01$, one way ANOVA) (Tables III.1 and III.8). Comparison among the 3 inbred strains showed C57BL/10 was significantly higher than the other two strains ($P<0.05$), but there was no difference between C3H/He and BALB/c mice ($P>0.05$) (GT2-test).

The DTH reaction was also tested in the congenic strains using a one-way ANOVA (Tables III.4 and III. 8). There was significant difference between the strains ($P<0.001$). B10.A was significantly different from three other strains (GT2-test). There was no correlation between oocyst production, mortality and the DTH reaction of all the tested mice.

Resistance to the Secondary Challenge. Following the challenge with 20,000 oocysts on day 32 after the first inoculation, the reinfected mice had a moderate degree of diarrhea on DPI 6-7 (much less than after the first infection in terms of duration and

Table III.8. ANOVA table: DTH response.

a). Adult group of inbred mouse strain.

source of variation	df	SS	F	P
Strain	2	337.05	6.79	0.0048
Error	23	570.83		
Total	25	907.88		

b). Congenic strains.

source of variation	df	SS	F	P
Strain	5	2312.02	8.72	0.0001
Error	44	2333.10		
Total	49	4645.12		

c). Comparison of significance among strains with GT-2 test (α=0.05 indicated by '***').

	C57BL/10	BALB/c
C3H/He	***	ns
C57BL/10		***

	B10.BR	B10.D2	C57BL/10	B10.A	B10.M
B10.PL	ns	ns	ns	ns	ns
B10.BR		ns	ns	***	ns
B10.D2			ns	***	ns
C57BL/10				***	ns
B10.A					ns

severeness). Oocyst production was greatly reduced (95% less, for data see Fig VII.4) from the primary oocyst production ($P<0.001$). Although the oocyst production was reduced, the prepatent period was still the same as during the first infection.

IV. CHARACTERIZATION OF MURINE DELAYED-TYPE HYPERSENSITIVITY RESPONSE TO COCCIDIAN INFECTION

In the previous chapter mice infected with *Eimeria falciformis* showed a footpad swelling response to injection of oocyst antigen (OA) which was proposed as a delayed-type hypersensitivity response. Is the response a true DTH? The following experiments were designed to test this hypothesis.

Murine DTH Pattern to *Eimeria falciformis* Infection. The pattern of DTH reactivity in hyperimmune mice with *E. falciformis* is shown in Figure IV.1. For this experiment eight C57BL/6 mice were each infected with a primary inoculation of 500 sporulated oocysts followed by a challenge of 20,000 oocysts on 30 DPI. The DTH response level was measured at hourly intervals (0-8hr), bihourly (10-24hr) and then at 30, 34, 48, 60, and 72 hr after OA challenge into the footpad. The first swelling peak occurred 2 hr after antigen challenge and then receded. Twenty-four to 48 hr after antigen challenge the second peak of swelling occurred. This response fits the classical DTH response as described by Loveren and Askenase (1984) who found that classical 24 to 48 hr DTH skin reactivity was preceded by an early T cell and tissue mast cell dependent swelling reaction which peaked 2 hr after antigen challenge. This pattern is clearly different from an Arthus reaction which is antibody mediated and reaches a peak swelling at 4-10 hr after antigen challenge (Roitt et al., 1985).

The DTH reaction was detectable three months after the first infection. The DTH was also OA specific. Injection of sheep red blood cells as nonspecific control did not induce footpad swelling at 48 hr after the injection, while OA induced specific swelling.

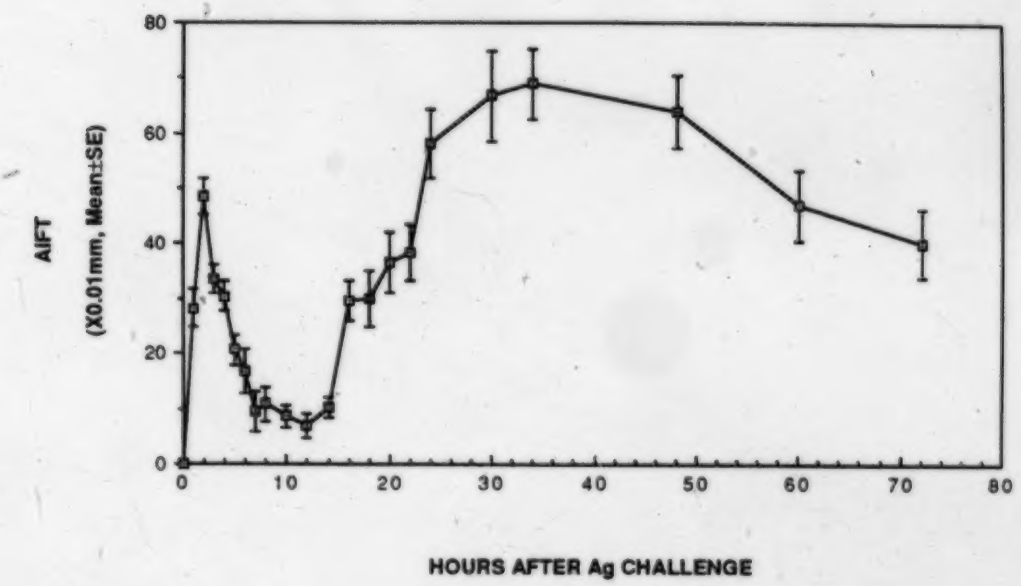


Fig. IV.1. Time course of DTH skin reactivity of C57BL/6 mice to oocyst antigen one week after the recovery from the second infection with *Eimeria falciformis* (data from 8 mice).

This difference was highly significant (P<0.001) (Table IV.1).

Phenotype of the Cells Mediating DTH to Oocyst Antigen. To determine whether OA-induced DTH was mediated by immune cells, unfractionated immune spleen cells from hyperimmune donor mice were transferred to syngeneic mice. Four hr after cell transfer, the recipient mice were infected with 500 sporulated oocysts. DTH reactivity to OA was induced by injecting 8,000 shattered oocysts into the footpad at different time points after cell transfer and measured in terms of 24 hr footpad swelling response (Table IV.2). Four hours after cell transfer recipients showed a significant DTH reaction (P<0.01) (group A versus B). During the peak oocyst production period (10 DPI) the DTH response in animals receiving immune spleen cells (ISC) was not different than in control mice (Group C versus D). It is noteworthy that in animals receiving immune cells, DTH was significantly lower on day 10 than when tested four hr after cell transfer (group A), as well as when tested on day 22 after infection (group E). Twenty-two days after infection recipients of immune spleen cells showed a significantly higher DTH than control mice (group E versus F). These data suggest that: 1) the murine DTH reaction to *E. falciformis* is transferable with immune spleen cells; 2) during the peak oocyst production period (day 10), DTH reactivity is temporarily suppressed; and 3) the transfer of immune spleen cells improves the post-infection DTH reaction (day 22).

To evaluate the contribution of immune spleen T cells in the adoptive transfer of DTH, spleen cells from hyperimmune mice were passed through anti-Ig:Ig columns to deplete Ig-positive cells(Wigzell, 1976). The Ig-negative (T cell enriched) cells were injected intravenously into syngeneic, naive mice. Twenty-four hr after cell transfer, DTH was measured. The results shown in Fig. IV.2, demonstrate that the T cell enriched population transferred the DTH reactivity. Thy-1$^-$ spleen cell recipients showed no DTH response (Fig. IV.3). Our data demonstrate that T cells from immune spleen were

Table IV.1. The DTH response of mice three months after the first infection and antigen specificity test.

Antigen	Immune status	N	48 hr FT (Mean±SE)[a]
OA[b]	3 months PFI[d]	6	48.3±3.7
OA	After 3 infections	7	77.0±10.3
SRBC[c]	After 3 Infections	7	8.7±2.0***[e]

a: Footpad thickness in 0.01mm.
b: Oocyst antigen.
c: Sheep red blood cells (20ul of 25% dilution).
d: Post the first infection.
e: Significantly lower than oocyst antigen group (t-test).

Table IV.2. The time course of adoptive transfer of C57BL/6 mouse DTH to *E. falciformis* with immune spleen cells.

Group	N	Cell Transfer[a]	DTH Testing Time[b]	Mean 24hr DTH Level (0.01mm)	P[c]
A	4	+	4 hr	41.3±6.4	
B	4	-	4 hr	11.1±3.6	<0.01#
C	4	+	10 day	17.3±3.3	<0.05£
D	3	-	10 day	17.0±2.3	>0.05*
E	4	+	22 day	94.7±12.4	<0.001¥
F	5	-	22 day	52.3±4.6	<0.001§

a. $3X10^7$ immune spleen cells injected into each mouse of groups A, C, E.

b. Animals were infected four hr after cell transfer with 300 sporulated oocysts and tested for DTH four hr, 10 days or 22 days after cell transfer by injection of 8,000 shattered oocysts into the footpad. Footpad swelling was measured 24 hr after footpad challenge.

c. The comparison of DTH reactivity between groups was tested with t-test.

#: Between A & B.

£: Between A & C.

*: Between C & D.

§: Between E & F.

¥: Between C & E.

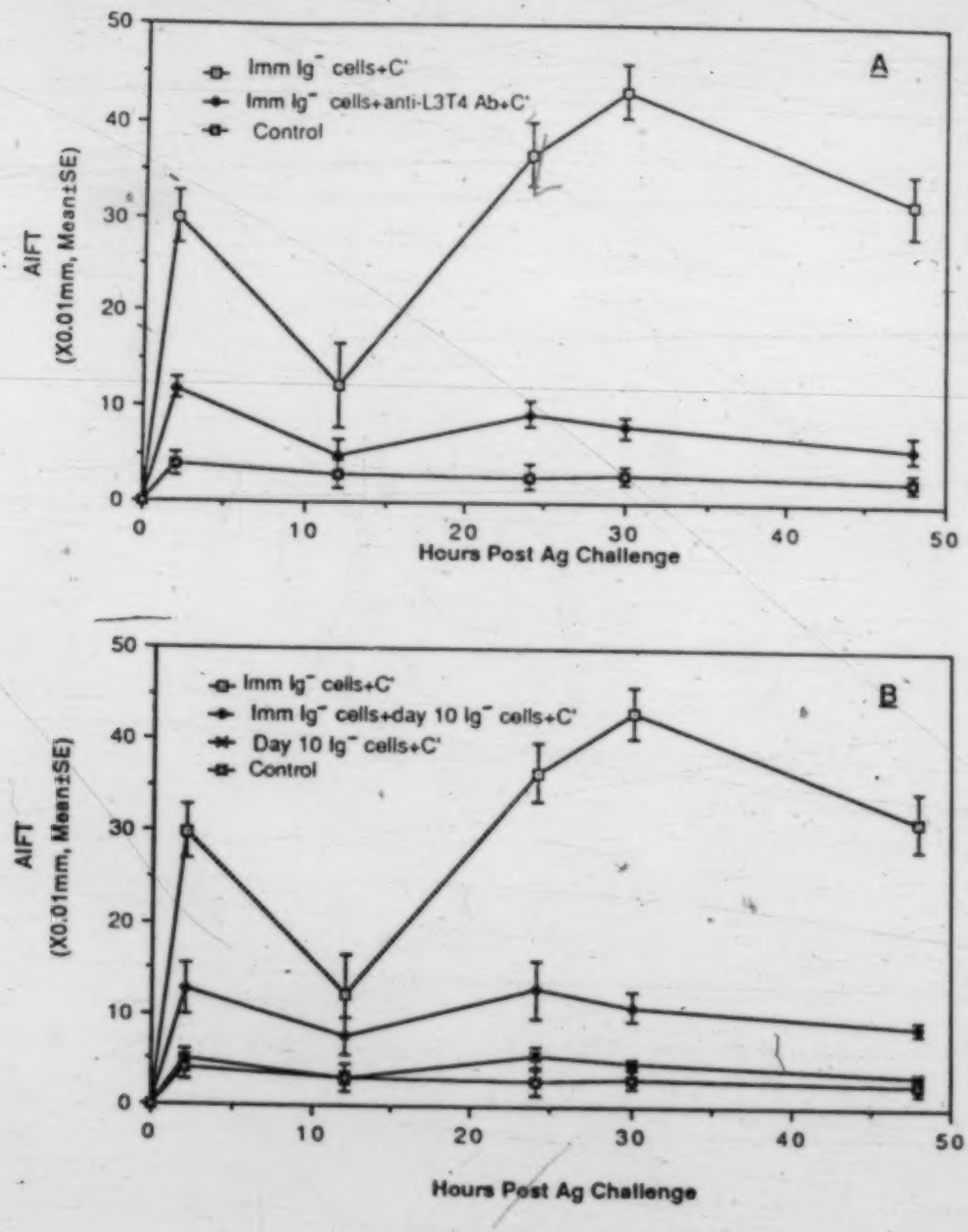


Fig. IV.2. The effect of anti-L3T4 antibody on adoptive transfer of DTH by T cell enriched immune spleen cells and suppression on T cell enriched spleen cells from acute infected (10 days after infection) mice on the adoptive transfer of DTH by T cell enriched immune spleen cells.

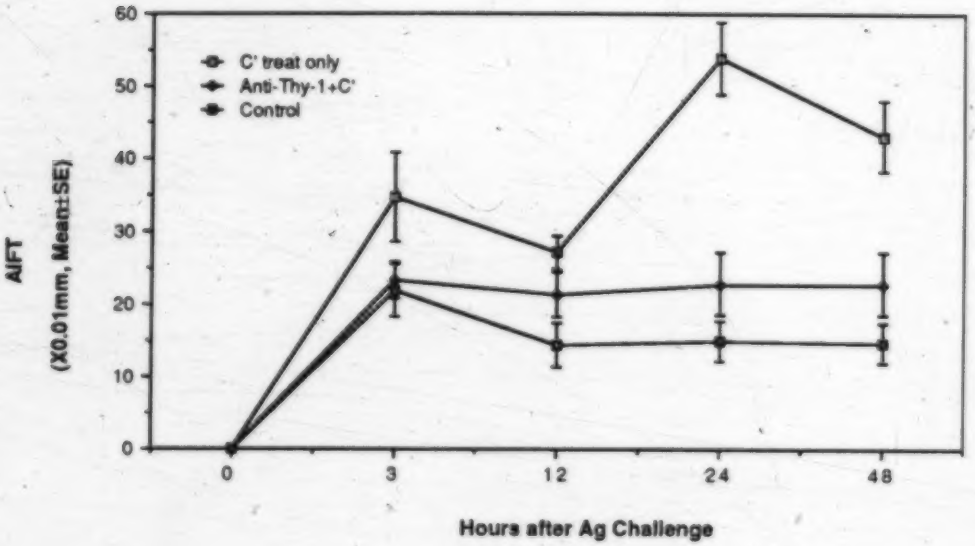


Fig. IV.3. The effects of anti-Thy-1 on immune spleen cell on transferring DTH to *Eimeria falciformis*.

responsible for DTH transfer.

The requirement of L3T4$^+$ cells in the adoptive transfer of DTH was investigated by treating Ig-negative spleen cells with anti-L3T4 antibody and complement. The recipients of immune L3T4$^-$, Ig-negative spleen cells showed no DTH response while recipients of complement treated Ig negative spleen cells exhibit a strong DTH response(Fig. IV.2). Thus, the L3T4$^+$ T cells were the responsible population in the transfer of DTH.

Abrogation of Transferred DTH by Spleen Cells from Acute Infected Mice. In the previous section we demonstrated that immune cell recipients lost their DTH reactivity 10 days after infection (acute period). To investigate the possibility that the parasite induced suppressor cells in these mice, spleen cells prepared from mice 10 days after the first infection were transferred together with immune spleen cells (Fig. IV.4). Mice receiving immune spleen cells (ISC) showed good DTH, while those mice receiving Ig-negative spleen cells from acutely infected mice showed no DTH. When Ig-negative immune spleen cells were transferred with Ig-negative spleen cells from mice infected for 10 days, the DTH transferred with Ig-negative ISC was abrogated (Fig. IV.2).. Thus spleen cells from acutely infected mice suppress the DTH transferred by immune spleen cells.

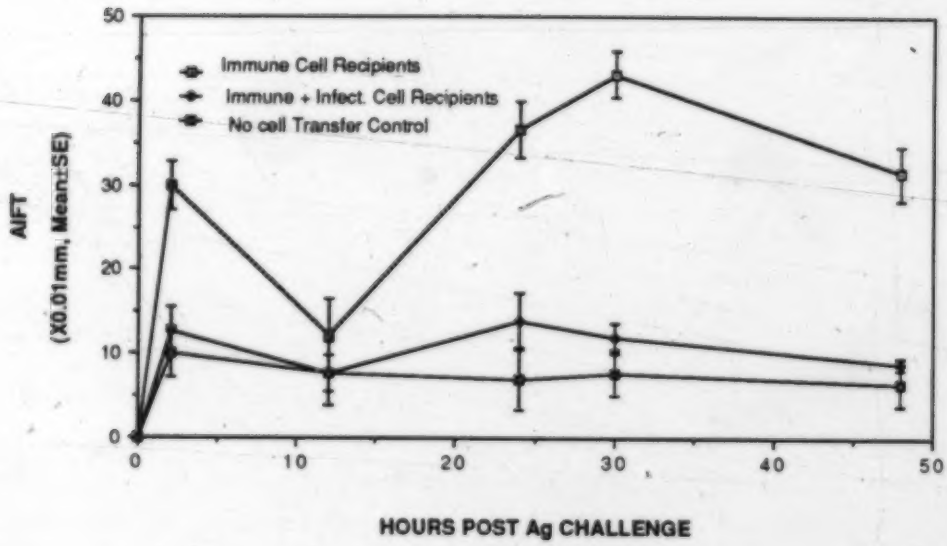


Fig. IV.4. The DTH response to OA transferred with $3X10^7$ immune spleen cells can be suppressed by spleen cells $3X10^7$ spleen cells from mice 10 days after the primary infection with 500 sporulated oocysts.

V. ADOPTIVE TRANSFER OF IMMUNITY TO *EIMERIA FALCIFORMIS* INFECTION

The experiments in this section were designed to determine if immunity to *Eimeria falciformis* could be transferred with lymphocytes from immunized mice to naive syngeneic mice. Since the 1970's adoptive transfer of immunity with lymphocytes to eimerian infections has been reported with some success.

Six experiments were conducted in this section. The results are presented in Tables V.1 and V.2. Data in these experiments were analysed with two-way ANOVA with treatment and fecal collection date as the two factors (Table V.3). In experiment 1 different number of immune spleen cells were transferred into syngenic naive recipients. The oocyst production in the five experimental groups was not different ($P>0.05$). This might be due to small sample size. In experiment 2 when larger sample sizes were used there was a significant difference between the experimental group and the control group ($P<0.001$). Immune spleen cells transferred immunity to infection with *E. falciformis*, as measured by total oocyst production. In the third experiment mice were infected at one of four different time points either before or after immune spleen cell transfer. All mice received equal number of cells. The oocyst production in these mice was significantly different ($P<0.05$). Multiple comparison (Tukey-test) showed that the group of mice infected 24 hr after cell transfer had significantly lower oocyst production than the other groups ($P<0.05$). In the fourth experiment Thy-1 negative cells were transferred as compared with whole spleen cell population transfer and control group. There was no difference in oocyst production ($P>0.05$).

In experiment 5 the recipient mice were sublethally irradiated with 500 rads. The oocyst production of the irradiated mice who received immune spleen cells was

Table V.1. Effect of Transfer of Immune Spleen Cells on Production of Oocysts of *E.falciformis* in C57BL/6 mice.

Expt. No.	Group No.	Recipients (N)		N[§] ($X10^7$)	T.O.P[‡] (Mean±SE, $X10^6$)
	1	4		5	10.41±2.13
	2	4		2	13.35±1.50
1[¥]	3	4		1	14.82±1.24
	4	4		0.5	13.41±1.61
	5	3		0	11.10±0.84
2[¥]	1	11		3	7.67±0.75***
	2	11		0	13.21±0.86
			Infection Time From Cell Transfer		
	1	5	+72hr	3	11.49±2.54
	2	5	+48hr	3	15.77±0.73
3	3	5	+24hr	3	9.74±0.87**
	4	5	-24hr	3	11.67±2.96
	5	5	Control	0	15.33±1.12
	6	5	Reinfection	0	0.40±0.11***

‡ Total oocyst production; Data were analysed according to the bidaily oocyst excretion with two-way ANOVA.

§ Number of spleen cells transferred.

¥ Infection with 300 sporulated oocysts four hr after cell transfer.

** Significance at $P<0.01$.

*** Significance at $P<0.001$.

Table V.2. The effect of transferring different spleen cell populations on the production of oocysts of *Eimeria falciformis* in C57BL/6 mice.

Expt. No.	Group No.	Recipients (N)	Mice & Cell Treatment	N$ ($X10^7$)	T.O.P‡ (Mean±SE, $X10^6$)
4	1	6	Spleen cell anti-Thy-1+C'	3	9.86±0.41
	2	6	Spleen cell+C'	3	9.54±0.80
	3	5	Control	0	10.84±1.54
5	1	6	No irrad.& no cell trans.		14.96±1.39
	2	7	No irrad.& cell trans.	3	12.71±0.92
	3	5	Irrad & cell trans.	3	9.64±2.51**
	4	5	Irrad & no cell trans.	0	16.18±2.02
6	1	9	Irrad & T cells enriched trans	1	13.17±0.85
	2	4	Irrad & spleen Cells	5.6	14.25±5.25
	3	9	Irrad & no cell trans.	0	13.46±1.50

‡ Total oocyst production; Data were analysed according to the bidaily oocyst excretion with two-way ANOVA.

$ Number of spleen cells transferred.

** Significance at $P<0.01$.

Table V.3. ANOVA tables: Adoptive transfer experiments.

1). Experiment 1.

Source of variation	df	SS	F	P
Treat	3	3.49	0.34	0.7937
Date	2	669.52	98.83	0.0001
Date*Cell dose	6	8.41	0.41	0.8645
Error	33	111.78		

2). Experiment 2.

Source of variation	df	SS	F	P
Treat	1	42.28	29.81	0.0001
Date	3	576.75	135.53	0.0001
Date*treat	3	45.13	10.60	0.0001
Error	80	113.48		

3). Experiment 3.

Source of variation	df	SS	F	P
Treat	4	30.29	2.74	0.0340
Date	3	1258.85	152.04	0.0001
Date*treat	12	72.18	2.18	0.0204
Error	80	220.80		

Table V.3. Continued.

4). Experiment 4.

Source of variation	df	SS	F	P
Treat	2	1.61	0.61	0.5498
Date	2	395.81	149.14	0.0001
Date*treat	4	1.53	0.29	0.8838
Error	42	55.73		

5). Experiment 5.

Source of variation	df	SS	F	P
Treat	1	26.62	4.35	0.0451
Date	3	341.60	18.60	0.0001
Date*treat	3	17.35	0.94	0.4305
Error	32	195.92		

6). Experiment 6.

Source of variation	df	SS	F	P
Treat	2	0.05	0.01	0.9938
Date	2	499.62	65.22	0.0001
Date*treat	4	8.70	0.57	0.6875
Error	36	137.88		

significantly different from those who were irradiated with no cell transfer ($P<0.05$). After recovery those mice were challenged with 2,000 sporulated oocysts on day 26 after the first infection. Irradiated mice with no cell transfer had significantly higher oocyst production than those with irradiation and cell adopted group ($P<0.001$) (Fig.V.1). Two of five mice died in the irradiated with no cell transfer group. In experiment 6, T cells and unfractionated spleen cells were transferred; no difference in oocyst production from the control group was detected.

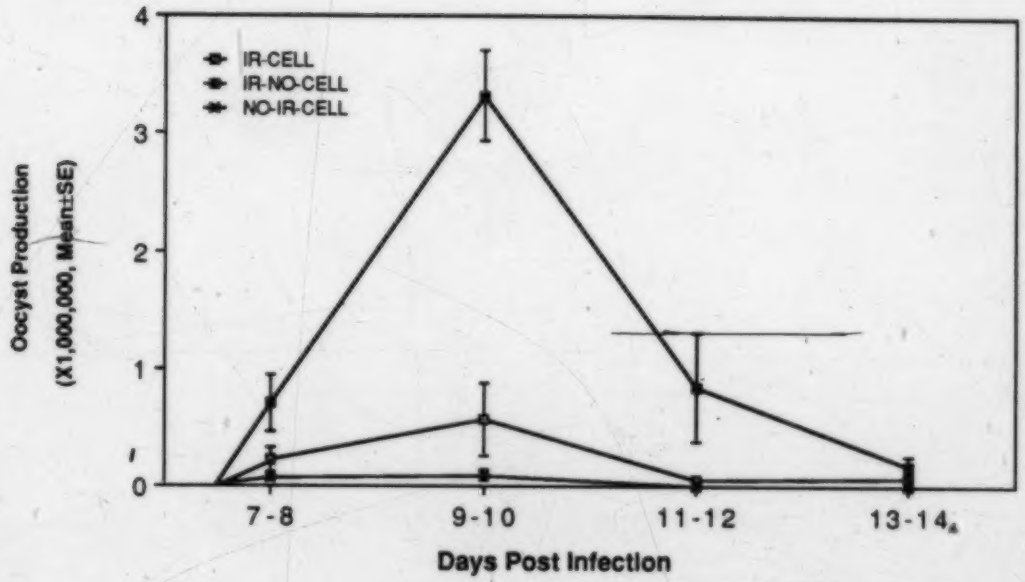


Fig. V.1. Oocyst production of secondarily infected irradiated C57BL/6 mice, with and without immune cell transfer. Each group had six mice, each was challenged with 2,000 sporulated oocysts 26 days after the first infection with 300 sporulated oocysts. IR-CELL=irradiation and cell transfer; IR-NO-CELL=irradiation without cell transfer; NO-IR-CELL= no irradiation with cell transfer.

VI. *IN VITRO* STIMULATION OF PRIMED LYMPHOCYTES WITH *EIMERIA FALCIFORMIS* ANTIGEN

The use of *in vitro* culture techniques is a powerful tool for measuring lymphocyte proliferation after antigenic, mitogenic or cellular stimulation (Mishell, 1980). The host immune response to coccidian infection has demonstrated a good DTH reactivity. Can lymphocytes from the immunized mice be stimulated with parasitic antigen to proliferate in an *in vitro* system?

Three preliminary *in vitro* culture experiments were conducted. In experiment 1 different concentrations of both oocyst antigen and spleens cells were used. The proliferation rate [count per minute (CPM)] depended on both Ag concentrations and spleen cell numbers. The resulting model explained 89.6% of variation in CPM and is highly significant ($P<0.001$) (Fig. VI.1).

The results in experiment 1 indicated: a) oocyst Ag has the capability to stimulate immune spleen cells to proliferate. b) the proliferation rate was closely dependent on number of spleen cells and oocyst Ag concentration; c) the Ag concentration of 5,000 oocysts stimulated the peak proliferation rate in spleen cells.

In experiment 2 three different parasitic antigens at two concentrations, and two concentrations of spleen cells were tested (Fig. VI.2). The proliferation rate of spleen cells tested with three parasite antigens was analyzed by 3-way ANOVA (Table VI.1). There were significant differences among Ag's, between Ag concentrations and between cell concentrations (all P's<0.001). GT2-test showed that oocyst Ag had significantly stronger ability to stimulate spleen cells than either oocyst wall antigen or merozoite antigen ($P<0.05$). There was no difference between oocyst wall antigen and merozoite

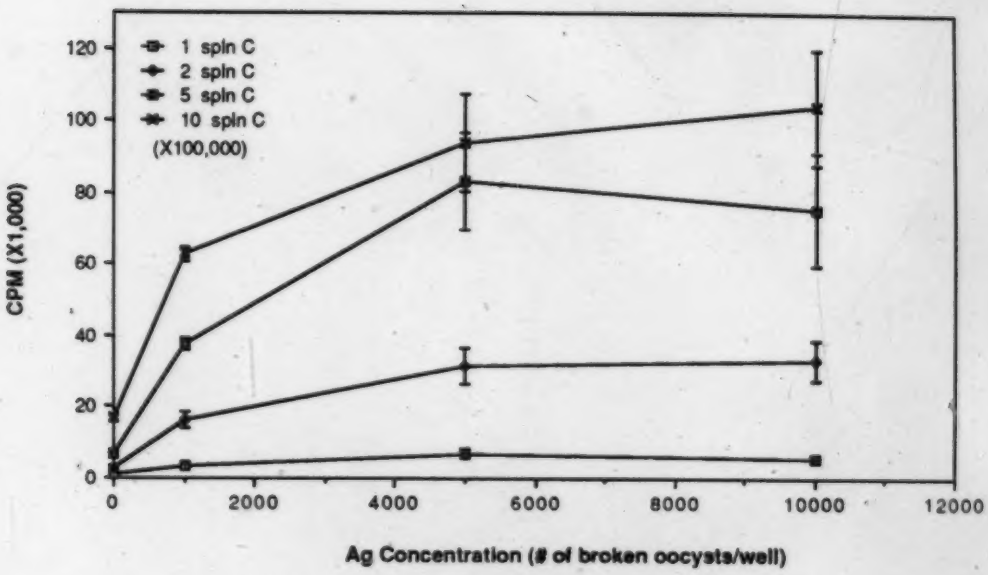
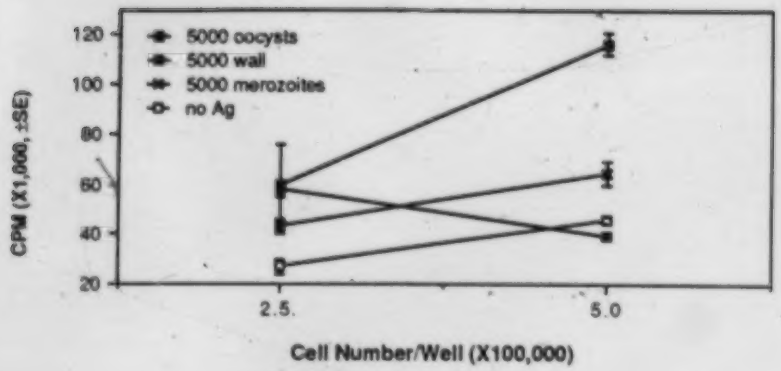


Fig. VI.1. *In vitro* stimulation of immune spleen cells by oocyst Ag. Spleen cells were harvested from recovered mice one month after infection with 2,000 sporulated *Eimeria falciformis* oocysts. Cells were cultured with different concentration of shattered oocyst antigen. spln C=spleen cells.



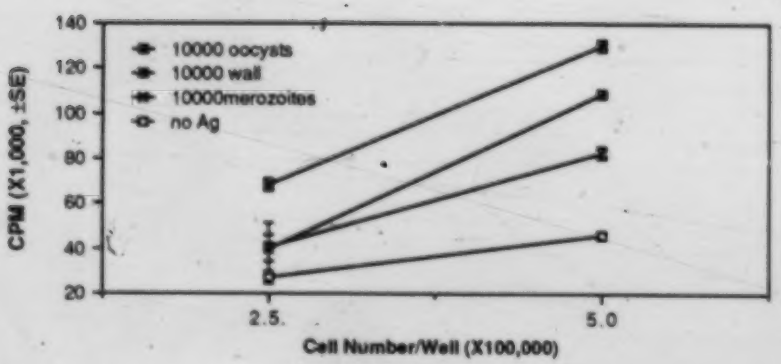


Fig.VI.2. *In vitro* proliferation of spleen cells with three types of parasite antigen. Spleen cells were harvested from immune mice which had been infected with 2,000 sporulated *Eimeria falciformis* oocysts.

Table. VI. 1. ANOVA table: Cultivation experiment 2.

Source of variation	df	SS	F	P
Antigen type (A)	2	12355375570.89	41.58	0.0001
Antigen amount (B)	1	3710887362.86	24.98	0.0001
Number of spleen cells (C)	1	12744473820.11	85.78	0.0001
A*B	2	733604510.69	2.27	0.1012
A*C	2	3756540884.89	12.64	0.0001
B*C	1	2700906906.77	18.18	0.0002
A*B*C	2	2693971890.26	9.07	0.0008
Error	31	4605652164.00		

Table VI.2. ANOVA table: Cultivation experiment 3.

Source variation	df	SS	F	P
Antigen treat (A)	1	1334603330.70	98.88	0.0001
Cell type (B)	1	186036375.15	13.78	0.0023
Cell number (C)	1	413318456.33	30.62	0.0001
A*B	1	220774846.26	16.36	0.0012
A*C	1	152182896.33	11.28	0.0047
B*C	1	13654755.59	1.01	0.3316
A*B*C	1	1992675.00	0.15	0.7066
Error	14	188951880.67		

Ag ($P>0.05$). No explanation can be given for the reduction of proliferation of cells cultured at higher cell numbers with oocyst wall antigen.

In experiment 3 two populations of immune spleen cells were cultured with different concentrations of oocyst antigen (Fig. VI.3). The results were analyzed with 3-way ANOVA by using Ag treatment, cell number and cell types as the three variables (table VI.2). Ag significantly increased cell proliferation ($P<0.001$). Higher number of cells introduced into cultures also increased cell proliferation ($P<0.001$). There was a significant difference between Thy-1 positive and negative spleen cells ($P<0.05$).

This experiment confirmed the antigen dependency of spleen cell proliferation that was demonstrated in experiment 1. The proliferation was also dependent on the number of spleen cells presented. These results agree with data from experiment 1. Experiment 3 also showed that lymphocytes treated with anti-Thy-1 reduced oocyst antigen stimulated *in vitro* proliferation.

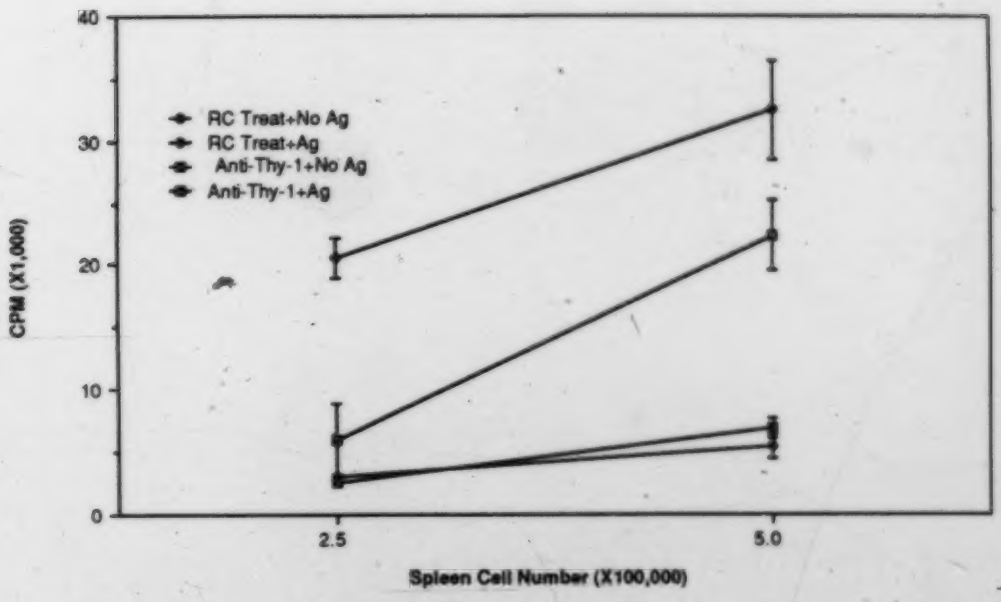


Fig. VI.3. The effects of anti-Thy-1 antibody on oocyst antigen stimulated in vitro spleen cell proliferation. Spleen cells were harvested from mice which had been infected twice, first with 1,000 oocysts and later challenged on day 30 with 20,000 sporulated *Eimeria falciformis* oocysts. Some spleen cells were treated with anti-Thy-1 monoclonal antibody and rabbit complement (RC), others were treated with rabbit complement only.

VII. ANTIBODY RESPONSE IN MURINE COCCIDIAN INFECTION

Do mice infected with *Eimeria falciformis* demonstrate a humoral immune response? To answer this question sera from infected mice were examined with an enzyme linked immunosorbent assay (ELISA), and antibody secreting cells were assayed with enzyme linked immunospot assay (ELISPOT). Antibody dynamics after the first and second infections were elucidated. Differences among classes and subclasses of immunoglobulins in three inbred strains were also tested. Although ELISA is a routine technique it has been difficult to compare the concentration of immunoglobulins of different classes and subclasses. In these experiments comparisons between classes and subclasses were made with myeloma immunoglobulin standards.

Calculation of Serum Antibody Concentration. An example of a standard curve is shown in Fig. VII.1. The standard correlation of absorbance, and the antibody concentration depended on at least four values selected from the straight portion of the standard curve (Fig. VII.2). The correlation equation was used for calculating test serum antibody concentration. The "X" value in the equation was obtained from straight portion of the testing curve (absorbance between 0.050-0.200) as indicated by the arrow in Fig. VII.3.

Dynamics of Serum IgG after Primary and Secondary Infection with *Eimeria Falciformis*. C57BL/6 mice were infected first with 1,000 and 35 days later with 20,000 sporulated oocysts. Oocyst production after the second infection was very low, more than 95% reduced than that from the first infection (Fig. VII.4). IgG first became detectable (with 20X dilution) eight days after the first infection. The amount of IgG

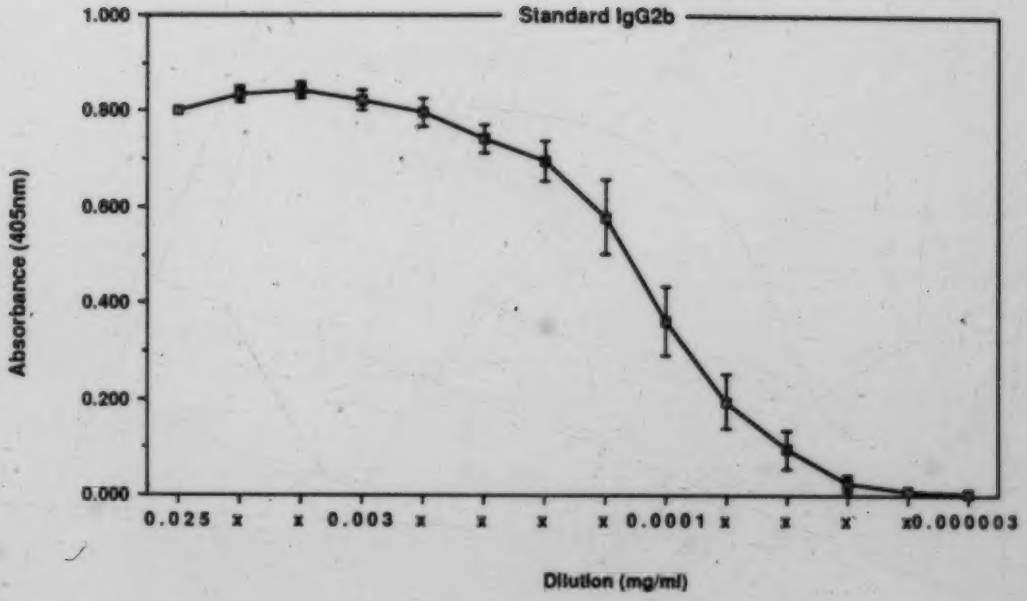


Fig.VII.1. Standard myeloma IgG2b titration curve. Detailed methodology is in text. Each point in the graph represents 5 samples. "x" indicates intermediate dilution.

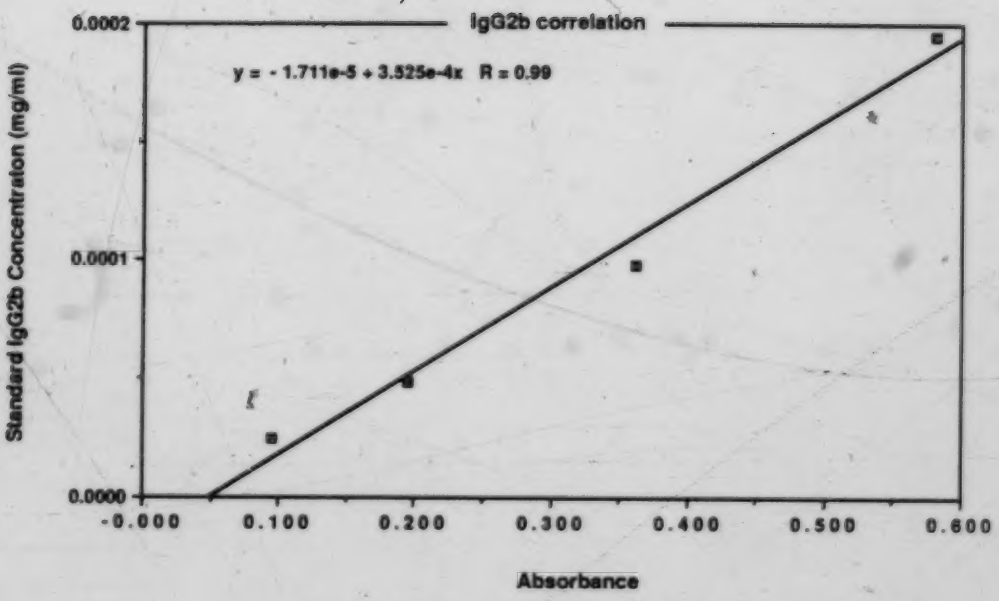


Fig.VII.2. Correlation of absorbance and the concentration of standard IgG2b. The four points in the graph were selected from the standard curve in Fig. 1. The formula is the simple correlation equation and was used to calculate the concentration.

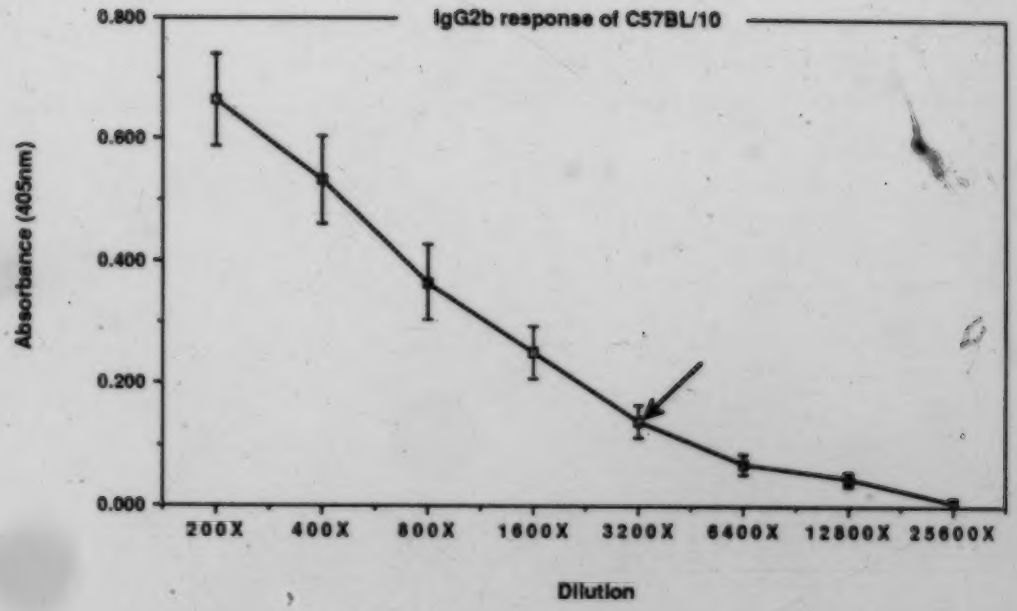


Fig.VII.3. IgG2b response of C57BL/10 mice. Arrow indicates the point selected for concentration determination with the equation in Fig. 2. The dilution factor for this point is 3200 times.

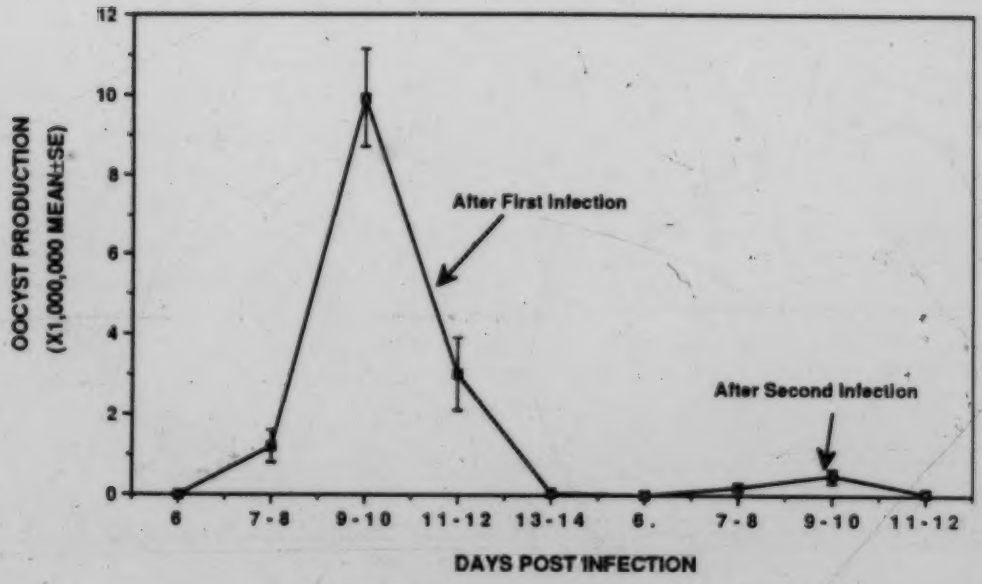


Fig.VII.4. Oocyst production in C57BL/6 mice each inoculated when 60 days old with a first infection of 1,000, and 30 days later with 20,000 sporulated *Eimeria falciformis* oocysts. Oocyst numbers were counted bidaily in individual fecal samples starting 7 days after inoculation. Each point present the mean oocyst production of 6 mice.

progressively increased, but it did not approach the response to the second infection (Fig. VII.5). A high IgG response was evident only 4 days after rechallenge and it persisted for at least 30 days.

IgA and Subclasses of IgG Response in Three Inbred Strains of Mice. Serum from C57BL/10, C3H/He and BALB/c mice in the first experiment (Table III.1) were collected on day 30 after the first infection. The results were analyzed by a two way ANOVA with strains and antibody classes as the two factors (Table VII.1). The results are shown in Fig. VII.6. Overall there were significant differences both between strains and between antibody classes ($P<0.001$). The significant interaction between strains and antibody classes ($P<0.001$) indicated that different class of antibody in different strains had different responses. IgA has generally been considered the most important antibody to gastrointestinal parasitic infection (Rose, 1982), however none of the three mouse strains had a pronounced IgA response. C3H/He had a very good IgG2a response, while C57BL/10 had no measurable IgG2a levels. C57BL/10 had a better IgG3 response than either of the other strains. None of the three tested strains showed an IgG1 response. There was no difference between the 3 inbred strains in absorbance in IgM production between the three inbred strains in relative terms (Fig. VII.7). Differences could not be quantified because an IgM standard was not available.

If the concentration of antibody response in all classes tested are considered, the response level can be listed as: C3H/He>C57BL/10>BALB/c. If the death rate (see chapter III) and antibody production are combined together, a positive correlation can be detected, indicating that antibody response was not effective in resistance to this coccidian infection.

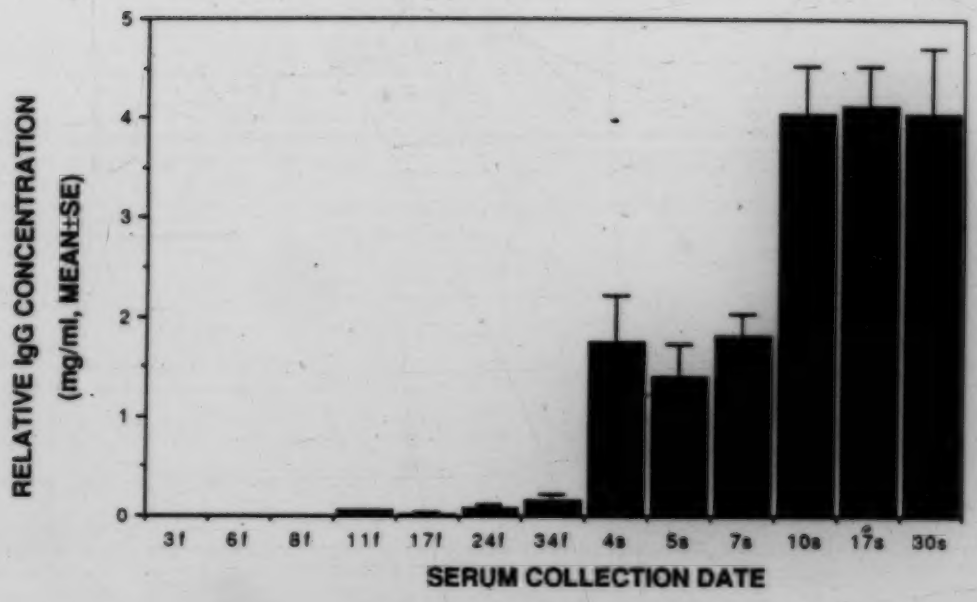


Fig.VII.5. IgG dynamics (detected by ELISA) to soluble oocyst antigen of *Eimeria falciformis* in serum of 6 C57BL/6 mice infected first with 1,000, 34 days later with 20,000 sporulated oocysts. "f" for days after the first infection. "s" for the days after the second infection.

Table VII.1. ANOVA table: Antibody concentration in three inbred mouse strains.

Source of variation	df	SS	F	P
Strains	2	0.7005	11.86	0.0002
Ab Classes	4	1.2135	10.27	0.0001
Strain*Ab class	8	2.6722	11.31	0.0001
Error	30	0.8860		

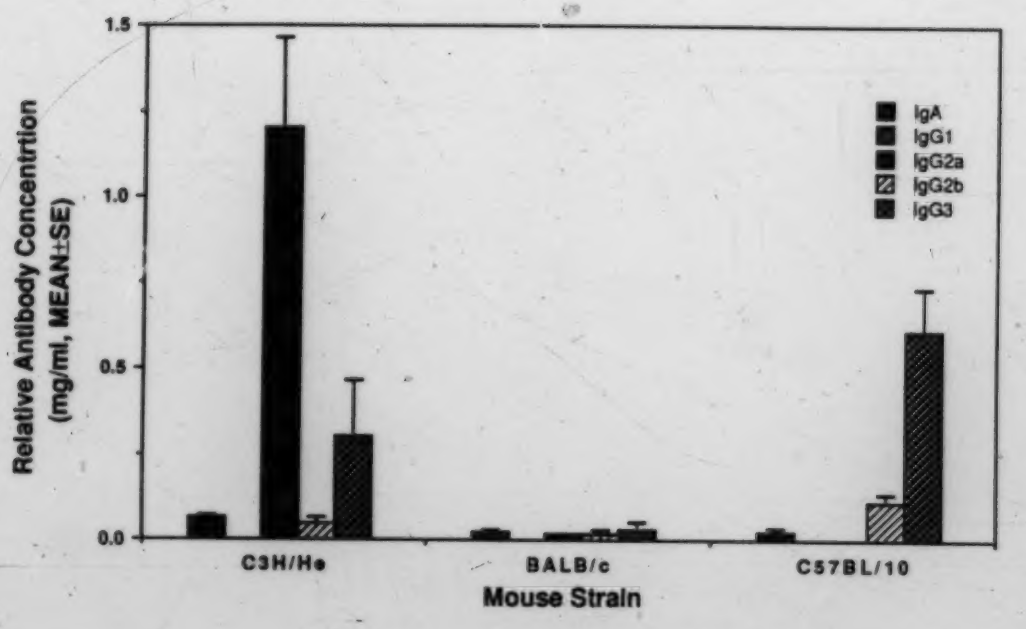


Fig.VII.6. IgA and subclasses of IgG response, detected by ELISA, in three inbred mouse strains (C57BL/10, C3H/He and BALB/c) 30 days post infection with 500 sporulated oocysts on day 0; 1000 on day 2 and 1500 on day 4.

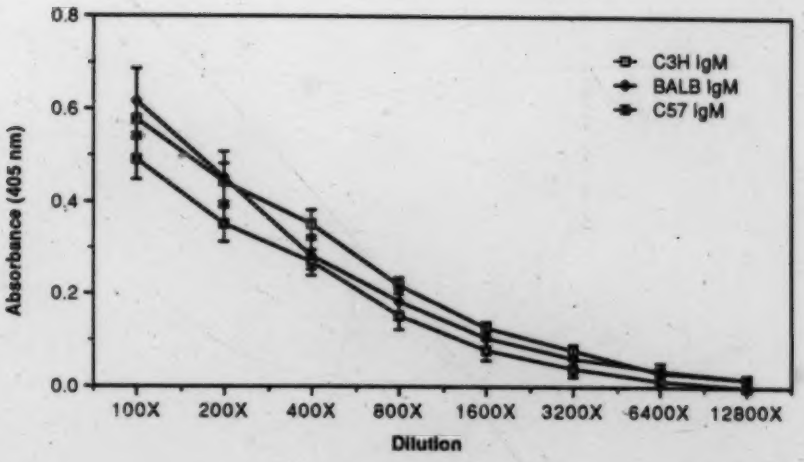


Fig. VII.7. IgM response in three inbred strains of mice. There was no significant difference in IgM response in the three inbred strains. Due to lack of standard IgM, no quantitative data are available.

The detection of IgG Secreting Cells in Secondary Lymphoid Organs. The ELISPOT assay technique is a new technique which is becoming increasingly important immunological research (Czerkinsky et al., 1987). Results using ELISPOT in our coccidia-mouse system indicate that the technique provides a reliable assay for determining the number of antibody secreting cells. This technique is antigen specific, antibody isotypic specific, and nearly background free as indicated by the control groups. There are about 25 IgG secreting cells in one million nucleated spleen cells of C57BL/6 mice nine days after a first infection, while in mesenteric lymph node cells from the same mice only eight out of one million nucleated cells secreted soluble oocyst antigen specific IgG (Table VII.2).

Table VII.2. ELISPOT assay for soluble oocyst antigen specific IgG secreting cells in spleens and mesenteric lymph nodes from C57BL/6 mice, infected first with 500 and 30 days later with 20,000 sporulated oocysts.

Cell Type	Petri Dish No.	Cells	Spots
Immune Spleen Cells	1	$3X10^6$	76
	2	$3X10^5$	8
	3	$3X10^4$	0
	4	$3X10^3$	0
	5	$3X10^2$	0
	*6 (no Ag)	$3X10^6$	0
Immune MLN** Cells	7	$3X10^6$	24
	8	$3X10^5$	2
	9	$3X10^4$	1
	10	$3X10^3$	0
	11	$3X10^2$	0
	*12 (no Ag)	$3X10^6$	1
No Cell Controls	*13	No Cell	0
	*14	No Cell	0
Non-immune Spleen Cells¥	*15	$3X10^6$	0
	*16	$3X10^6$	0

* Control groups.
** MLN=mesenteric lymph node.
¥ From specific pathogen free C57BL/6 mice.

VIII. DISCUSSION

A. Introduction

One of the most important mechanisms regulating host-parasite interaction is the host immune response. The murine coccidian system used in this study provides a good model for studying the homeostasis between host and parasite. Several aspects of host immunity to parasitic infection were investigated in this thesis using this murine coccidium model. The discussion of host immunobiology has been organized around several questions. How does the host genetic background influence resistance and susceptibility? Is oocyst antigen induced footpad swelling a true DTH response? Can T cells adoptively transfer immunity to coccidian infection? Can the immune response of T cells to the parasite be studied in vitro? How do antibodies respond to coccidian infection?

B. Genetic Control of Murine Immune Response to *Eimeria falciformis*

The experiment with three inbred strains showed marked differences in the susceptibility of different mouse strains to primary infection with *Eimeria falciformis*. The oocyst production varied significantly. C57BL/10 produced more oocysts than BALB/c, while BALB/c produced more oocysts than C3H/He. Klesius and Hinds (1979) compared the susceptibility of several mouse strains to *E. ferrisi* (this species also parasitizes the mouse large intestine). They also found that BALB/c mice were more resistant than C57BL/10 mice. However, all mice were uniformly able to resist reinfection. Although my results for *E. falciformis* were very similar to *E. ferrisi*, I cannot conclude that variation in susceptibility to eimerian infection in different mouse strains was the same for all *Eimeria* species in mice. Immune response genes are highly

specific in their methods of regulating immunity to different diseases (Mengle-Gaw and Mcpevitt, 1985). This is also true in chicken resistance to coccidiosis since some lines of chickens selected to resist some *Eimeria* spp. were more susceptible to other species (Patterson et al., 1961).

In the two genetic experiments carried out, no differences in life cycle were found even though oocyst production was significantly different. This agrees with Klesius and Hinds (1979) and Mesfin and Bellamy (1979), but differs from Rose et al. (1984) who found that high oocyst production strains correlated with prolonged patency in *E. vermiformis* infected mouse strains. Rose et al. (1984) suggested that additional cycle of merogony might be occurring in the high oocyst producing hosts. This may not be true, because most *Eimeria* spp. have a different number of merogonous cycles. The differences in the susceptibility to host response, and other environmental factors may precipitate differences in oocyst production by these species.

It is difficult to draw a clear conclusion regarding the function of major histocompatibility complex of mice (H-2) to infection with *E. falciformis*. Results from individual criteria were clear, but when relationships were sought among oocyst production, DTH, and mortality of the congenic strains (Table III.4), no clear correlations were found. The strains B10.BR, B10.D2, and B10.PL had greater mortality, showed lower DTH and had low to intermediate oocyst production (Mahrt and Shi, 1988). However, data from B10.A (which shares MHC genes with both B10.BR and B10.D2) contradicts the hypothesis that a part of the MHC controls these effects, since no correlation with either K, I or D region of the H-2 was detected. Some tentative conclusions can be reached:

a). Genes outside MHC also control susceptibility. The difference among congenic strains may not be associated with MHC since these strains may not be truly

congenic, and differences may be due to some other genes than MHC. No strain can be absolutely congenic (inbreeding coefficient equals 1.0) because of decreasing fertility and spontaneous mutation (Zaleski et al., 1983).

b). There can be interactions between loci in the MHC which control the expression of other genes. This is true for the I-J molecule which is proposed to be located outside MHC, while the expression is controlled by the class II genes (Murphy, 1987).

c). During the endogenous development of *E. falciformis* there are four asexual generations prior to oocyst formation. Researchers had explored the antigens of different parasitic stages of *Eimeria* spp. by using SDS-polyacylamide gel electrophoresis and western blotting (Kyle and Speer, 1984; Clarke et al., 1986; McAndrew et al., 1986), immunoprecipitation analysis (Wisher, 1983), ferritin labeling and transmission electromicroscopy (Speer et al., 1983), enzyme-linked immunosorbent assay (ELISA) (Rose and Mockett, 1983) and hybridoma antibodies (Danforth et al., 1986) in different coccidia species, and demonstrated extensive antigen cross reactivity and stage specific antigen specificity. At different stages parasites release different antigens to the immune system, and these antigens may interact differently with gene products of different immune response gene haplotypes. The different interactions may lead to different immune responses with different consequences for the host. Thus, the various criteria in the system need not be uniformly correlated.

Differences of DTH reactivity in congenic strains is very likely due to the influence of the association of MHC antigen with parasitic antigen. The development of DTH requires antigen presented in association with MHC class II Ag to T_{DTH} cells (Roitt et al., 1985). Different MHC antigens present antigen differently. This leads to the difference in DTH reactivity.

There were several different levels of oocyst production among congenic strains. Therefore, oocyst production was controlled by multiple genes. As indicated with the combination of the inbred strain experiment and the congenic experiment, oocyst production was controlled by genes both inside and outside the MHC. Hence, it is not surprising that differences occurred among strains. This phenomenon may also contribute to differences in oocyst production in this experimental system.

The absence of age-related differences in oocyst production may or may not indicate a difference in susceptibility to parasite protection (see Long, 1973 for review). There may be a difference in susceptibility, but adult mice have more epithelial cells for parasites to invade, while susceptible young mice have fewer epithelial cells.

Many other parasites such as *Giardia, Trypanosoma, Leishmania, Plasmodium , Trichinella* and *Schistosoma* are known to be host strain specific, and this specificity is obtained during the evolution of host and parasite interaction, which is important for host existence by having some individuals more resistant than others (see Mitchell et al., 1982 for review). MHC antigens play an important role in regulating the cellular interaction in the immune system. By carrying greater allotypic variation in MHC genes, hosts prevent parasites from escaping the immune system by imitating single molecules of the system. When a host population carrying different MHC haplotypes is examined, the immune system of each individual in a species has a different MHC haplotype. Hence, even the perfect pathogen can not evolve a strategy to spread through out a entire population. This is an important host evolutionary strategy (Roitt et al., 1985).

C57BL/10 had high oocyst production, high DTH reaction and intermediate death rate. These characters make this strain more sensitive to experimental treatments. Thus, C57BL/10 was the most suitable strain for further studies. C57BL/10 mice came from Jackson Laboratory, ME, U.S.A.. In order to save time and money in later experiments

C57BL/6, a genetically similar strain, was obtained from Charles River, Quebec, Canada.

Transgenic technique has been successfully applied to livestock such as pig, sheep and rabbit, which bears scientific importance and potential economic value (Hammer et al., 1985). Meur et al. (1985) transferred a cloned E_a^k gene into embryos of mice incapable of expressing their own endogenous E_a gene and therefore, non-responding to E-restricted Ag's. The transferred E_a^k were expressed in the new hosts and the hosts become responders to E-restricted Ag's. If we can identify the coccidian resistance gene from domestic animals and then transfer the resistance gene to an embryo, a resistant animal strain can be produced. Transgenic technique holds promise in the future for genetic control for coccidian infection.

C. Characterization of Murine Delayed Type Hypersensitivity Response to Coccidian Infection

Present experiments characterized the DTH response to *Eimeria falciformis*. The immune animals exhibited a classical DTH reaction. This conclusion is supported by the observation that the footpad swelling reaction peaks at 2 hours and also at 24-48 hours after footpad antigen challenge. The adoptive transfer of DTH reactivity with immune spleen cells can be abrogated with anti-Thy-1 antibody plus complement treatment. This suggests that Thy-1 positive T cells mediate DTH to OA. Similarly, removal of immunoglobulin-positive cells from immune spleens, and thus enriching for T lymphocytes, does not reduce the level of transferred DTH. Surface immunoglobulin negative immune spleen cells depleted of L3T4 positive cells were unable to transfer DTH to OA. Therefore, the major cell subset mediating classical DTH to OA consists of L3T4 positive T lymphocytes. The DTH response was oocyst antigen specific as shown by the negative response to sheep red blood cells.

During the acute stage of infection (i.e., peak oocyst production) animals were unable to mount a DTH response to OA. This existing suppression can prevent the adoptive transfer of DTH by immune spleen cells in acutely infected animals. Further more, the ability of immune spleen cells to adoptively transfer DTH to naive recipients is inhibited if spleen cells from acutely infected animals are cotransferred. The cells which mediate this inhibition of DTH by L3T4 immune cells are Ig negative, and may be either T lymphocytes or macrophages.

Cell mediated immunity (CMI) has long been considered as the basis for immunity to coccidia as shown by adoptive transfer of lymphocytes to syngenic or athymic and bursectomized animals (Liburd et al., 1973; Ogilive and Rose, 1977; Rose and Hesketh, 1982; Mesfin and Bellamy, 1979). Vaccine-induced immunity to *Eimeria* in chickens is also correlated with DTH (Giambrone et al., 1980). My experiments showed that CMI, as measured by DTH, may be associated with increased resistance against coccidiosis as evidenced by the ability of the transferred immune lymphocytes to reduce oocyst production, as well as prevent the death of recipients (Tables V.1 and V.2).

T cells can be divided into a Ly-2$^+$ (CD8$^+$) subpopulation and a L3T4$^+$ (CD4$^+$) subpopulation. The L3T4$^+$ T cells are proposed to have two primary functions. Firstly, L3T4$^+$ T cells cooperate with B cells, stimulating the latter to proliferate and differentiate into antibody secreting cells. Secondly, L3T4$^+$ T cells play a central role in the induction and elucidation of DTH (Klein, 1986). In addition L3T4$^+$ T cells can activate macrophages to destroy certain types of intracellular pathogens, such as in leishmaniasis (Liew, 1982). The murine L3T4$^+$ cells can be further divided into two populations, T_H1 (T helper cell 1) and T_H2 (T helper cell 2) according to the types of lymphokines produced. T_H1 is mainly responsible for DTH reaction and to a lesser extent help for B cells. T_H2 is mainly involved in helping B cells to produce antibodies (Mosmann et al.,

1986; Cher and Mosmann, 1987). Ly-2$^+$ T cells act as specific killer to target cells. Overall T cells give the host resistance to *Eimeria* infection (Rose, 1982) by some of the above functions, but the precise subpopulation involved is still not clear at present. Mogil (1985) demonstrated that both L3T4$^+$ and Ly-2$^+$ T cell subsets were involved in the protection to *Plasmodium yeolii* infection. I predict that the two subsets might be also involved in the resistance to *Eimeria* infections.

In other parasites and antigen systems, DTH reactivity is regulated by suppressor T cells (Liew, 1982; Bach et al., 1978; Miller and Jenkins, 1986). In my adoptive transfer experiments it was also apparent that, 10 days after infection, *Eimeria* can induce Ig$^-$ cell population mediated suppression to oocyst specific DTH. During the acute period of infection, proliferating parasites may release antigens which induce cells which then suppress the oocyst antigen specific DTH. Further studies are needed to clarify the role of parasite-derived productions in the induction of DTH, and how this suppression affects the subsequent establishment of immunity in the host. Immunosuppression in protozoan infections is an important adaptive mechanism to evade the host immune system (Bloom, 1979). Yano et al. (1987) demonstrated that during acute *Toxoplasma gondii* (a related coccidian parasite to *Eimeria*) infection, non-specific suppressor T cell can reduce lymphocyte *in vitro* proliferation.

It is possible that during the primary infection different degrees of immunosuppression occurred among strains, and these differences precipitated differences in susceptibility . During the recovery period from primary infection, mice may generate another population of T cells (T contrasuppressor cells) as demonstrated in gut associated lymphoid tissue of orally tolerized mice with artificial antigens. Such contrasuppression may have made every mouse strain resistant to reinfection. Green and Ptak (1986) suggested that antigen overload and tissue damage caused by proliferating

pathogens can suppress immunity. These mechanisms may be applicable to the murine coccidium model and need exploration.

All mice studied (both resistant and susceptible strains) were resistant to reinfection in terms of oocyst production. This is consistent with previous reports (Stockdale et al., 1985; Rose et al., 1984). However, mice which recovered from primary infection when reinfected still exhibited diarrhea, depression, rough hair and dehydration on DPI 8-10. Mesfin (1973) showed that even in immune mice, sporozoites and merozoites still penetrated epithelial cells of the colon and migrated to the crypts, but very few parasites completed their life cycle. In this study acutely infected mice showed immunosuppression to DTH response in the primary infection. This immunosuppression may also contribute to the induction of the clinic signs after the secondary infection. Or we can predict the immune response may be inhibiting development throughout the life cycle, but has its strongest effect on the last asexual generation and gametogony. To verify this hypothesis some histological work is needed.

D. Adoptive Transfer of Immunity to Eimeria falciformis Infection

In several parasitic infections, such as *Leishmania* and *Plasmodium*, it has been difficult to establish the role of antibody in protection, but cell mediated immunity (CMI) is believed to be fundamental (Allison and Eugui, 1983). Antigen activated T cells release cytokines to activate macrophages which destroy parasites as established in *Leishmania* infection (Louis et al., 1984). The early knowledge of the roles of cytokines in eimerian infections was obtained from the ability of transfer factors to increase recipient resistance to infection (Klesius et al., 1977; Liburd et al., 1972). Kogut (1987) and Speer et al. (1985) obtained Con-A induced lymphokines which suppressed parasite (*E. bovis and E. papillata*) growth in cell culture. Another role of T cells is to directly lyse infected cells by recognizing parasite antigen in association of host MHC class I antigen on the

infected cell surface. This has been shown to be the major mechanism in *Theileria* infection (Allison and Eugui, 1983). This mechanism may also be important in eimerian infections. An approach to this question would employ the chromium release assay (Simpson and Chandler, 1986).

Is there a positive correlation with the immune response to *Eimeria* infections? Although Giambrone et al. (1980) showed that DTH was correlated with coccidian immunity in chickens, my experiments show that CMI, as measured by the DTH response, was not always associated with increased resistance against coccidian infection, as evidenced by the inability of the transferred immune lymphocytes to reduce oocyst production in recipients in some experiments. The DTH reaction might not be a good parameter of CMI as shown by Hurtrel et al. (1981) where no correlation existed between DTH reactivity and the host susceptibility to candidiasis. Also the number of transferred cells in my adoptive transfer experiments might have been insufficient to adoptively transfer resistance to parasite development. Initiation of the DTH reaction may need fewer cells than required for resisting infection. Marchal et al. (1982) showed that DTH could be transferred locally with a single cloned T cell. Liburd et al. (1973) showed that rats were totally resistant to infection with *E. nieschulzi* only when high numbers (10^9 cells) of immune thoracic duct lymphocytes (TDLs) were transferred. Lower number of TDLs ($<10^8$ cells) did not significantly reduce oocyst production. In contrast, immune spleen cells in my experiments always effectively transferred DTH reaction to recipient mice. This indicated that fewer cells were needed for DTH transfer than for reducing oocyst production, however, the exact role of CMI would be best demonstrated with cell transfer systems.

The use of irradiation provided an opportunity for detecting the differences between experimental and control groups since irradiated recipients lose all lymphocytes,

thus, eliminating the interaction between transferred cells and host lymphocytes. The irradiated mice also become more susceptible to secondary challenge due to the loss of the ability to acquire immunity thus increasing the difference between control and experimental groups.

Two potentially useful techniques to determine the phenotype(s) of lymphocytes and their roles in immunoregulation are: 1). through adoptive transfer or, 2). using high dosage of anti-lymphocyte monoclonal antibodies. These techniques provide valid *in vivo* information concerning immune mechanisms.

E. *In Vitro* Stimulation of Primed Lymphocytes with *Eimeria falciformis* Antigens

In vitro culture methods have provided immunologists with new investigative techniques. Many *in vivo* phenomena have been duplicated *in vitro* systems. The introduction of growth factors into *in vitro* systems makes these techniques more applicable to biological sciences. For example, T cell growth factor (such as interleukin-2) made the *in vitro* culture of T lymphocyte lines practical, and greatly increased our knowledge of cellular immunoregulation (Fathman and Engleman, 1986).

The application of *in vitro* methods to coccidian infection research started in 1920's (Doran, 1973), and allowed research into the biology of the parasite. Recent research by Speer et al. (1983, 1985) has combined *in vitro* cell culture of coccidia and immunology. The effect of Ig classes and lymphokines on coccidia infected cells in culture were studied.

Coccidian antigens (whole oocyst antigen, oocyst wall antigen, and merozoite antigen) have the capability of stimulating immune spleen cells to proliferate. The proliferation rate was closely dependent on numbers of spleen cells and Ag

concentrations. Lymphocytes treated with Thy-1 affected oocyst antigen stimulated *in vitro* proliferation of spleen cells. These cell culture experiments were very preliminary. Since the cells were nearly unfractionated, no conclusion about which cell(s) were proliferating, or how the cells may have cooperated. Nevertheless, the results do indicate that the antigen specific immune response can be obtained in an *in vitro* system.

In the second *in vitro* experiment three types of antigen stimulated spleen cells to proliferate. Whole oocyst antigen showed the best ability to stimulate immune lymphocyte proliferation. Since oocysts are much larger than merozoites and each oocyst contains eight sporozoites, the results are not directly comparable. In future experiments protein concentrations instead of number of parasites should be used.

By using immunofluorescent antibodies, Mesfin (1973) demonstrated that antibodies did not bind to oocysts and sporocysts. He concluded that oocysts and sporocysts were unable to stimulate humoral immunity. In my *in vitro* system oocyst walls and whole oocyst antigens stimulated the lymphocytes to proliferate. Therefore, the present system provides some advantages over Mesfin's system for detecting parasite antigenicity; namely higher sensitivity, wider measurement of the spectrum of immunity (stimulating both T and B cells), and being semi-quantitative since DNA synthesis can be measured.

The *in vitro* system is useful for characterizing protective antigen(s) that induce protective immunity, and to study the roles of different lymphokines and different subsets of lymphocytes. For example, the chromium release technique could be used to study the role of cytotoxic cells (Simpson and Chandler, 1986). Overall, this system has good potential for the future studies of eimerian immunology.

F. Antibody Response in Murine Coccidian Infection

The role of antibody response in the immunity to *Eimeria falciformis* infection is uncertain. Mice produced a strong IgG response, especially after secondary infection. This contradicts Lillehoj and Ruff (1987) who found no significant increase in antibody production by chickens infected a second time. The role of IgG in the immune process remains unclear.

Lillehoj and Ruff (1987) found no difference in IgG and IgA production in the two chicken strains studied. The comparison of antibody production in the three inbred mouse strains of my experiments indicated some variations between different classes or subclasses of antibodies. Overall C57BL/10 produced more antibody than BALB/c, and C3H/He produced more than C57BL/10.

IgA has been traditionally considered an important antibody for parasitic infections of intestinal mucosa. IgA is a important antibody present in mucosal secretions (Jeske and Capra, 1984). The number of IgA containing cells and the concentration of IgA in the intestinal mucosal secretion increased in chickens after infection with *E. tenella* (Davis et al.,1978). Further Rose and Mockett (1983) showed there was an IgA response to *Eimeria* infection. In my experiments little IgA was found in serum as compared with other antibody classes. Rose and Mockett (1983) found a very strong IgA response in chickens infected with *E. tenella* in both intestinal washings and bile. In future investigations, more extensive use of the ELISPOT technique to test the IgA secreting cells from the Peyer's patches, should give a clearer understanding about the role of IgA in eimerian infections.

The three mouse strains showed an absence of IgG1 response. C57BL/10 had no Ig2a response while C3H/He had a strong IgG2a response. These differences were probably due to host genetic differences. The role of different subclasses of antibodies in the development of protective immunity to coccidian infection remains to be investigated.

Differences in production of different classes of antibodies suggest that there may be relatively few kinds of antigen responsible for antibody production. Since each antigen tends to favor a specific class of antibody, the presence of many antigens would decrease the likelihood of finding large differences between classes of antibodies. If only a few antigens are involved, the likelihood of large differences would increase.

It was difficult to correlate the criteria used in this study. However, in inbred strains the death rate was positively correlated with overall immunoglobulin production, however, this correlation is difficult to interpret at this time. The role of antibodies in immunity to coccidian infection is still unknown (Lillehoj and Ruff, 1987), but likely play a minor function compared to cell mediated immunity (Rose, 1982). The effects of bursectomy in chickens suggested that antibodies were not critical for immunity (Lillehoj et al., 1986). Before making a definitive conclusion about the importance of antibodies in coccidian infection further experiments on immunity of both avian and mammalian hosts are needed.

ELISA is a sensitive assay for detecting antibodies in body fluids and in *in vitro* culture systems. Due to the simplicity and the availability of the reagents, ELISA is a widely used technique. It offers a very good comparison of the concentration of the same class or subclasses in different experimental samples. However, comparisons between classes or subclasses of antibodies have been troublesome due to differences of affinity to antigens among different classes of antibody. In this thesis a technique was established using immunoglobulin standards which made more precise comparison. Since the examined serum antibody bound to the plastic plate surface through recognition of coated antigens, and the immunoglobulin standards were directly attached to the plastic surface, error may have been introduced as antigen and immunoglobulin may have different binding affinity to the plastic surface. With further experiments, a correction factor could

be established which could give more quantitative measurements. Because differences in oocyst production were detectable only after the first infection and all strains were resistant to the second infection, antibody production from only primary infections was compared.

The enzyme linked immunospot assay (ELISPOT) technique indicated that the spleen had more antibody secreting cells than the mesenteric lymph node. These were preliminary results which need duplication. ELISPOT can quantitatively test the number of cells secreting a specific class or subclass of antibody in a given cell population from either a specific organ or from a cultured cell line. This technique is an important development which can be applied to immunoparasitology since most parasites only live in a specific region in the host. The local immune response may be much more important than the systemic reaction.

G. Future Prospects

The murine coccidian system in this study gave very promising results for further studies. This system can not only give fundamental knowledge leading to the control of coccidiosis, but also can be a suitable model for studying gastrointestinal immunity to other pathogens.

The immune mechanism in the gastrointestinal tract differs from that of other systems (Bienenstock and Befus, 1985). In recent years gastrointestinal immunology has become an active and attractive area to immunologists, nutrition scientists, clinical doctors, and many others. It has revealed some exciting results important to fundamental immunology (e.g., immunoregulation) and clinical medicine (e.g., gastrointestinal infection, food allergy and oral vaccination). Most research in this area has used noninfectious antigens, which most likely do not act the same as living infectious agents.

The murine coccidian parasite system can be readily used as a model to study how infectious antigens act in the intestine, and are recognized by the host immune system. The following aspects of coccidian immunity need further investigation.

1). How is the immune system and gut associated lymphoid tissue regulated in coccidian infection?

2). Which cell population (s) play fundamental roles in protective immunity?

3). How do cells in the immune system cooperate in the immune process?

4). Which antigen(s) from which parasitic stage are important in immune induction?

5). How do lymphokines affect the infection, and what role do they play?

6). What role does IgA play in this intestinal infection?

7). What are the functions of macrophages in direct killing and in antigen presentation?

At present, eimerian antigens are being characterized with monoclonal antibodies (Danforth et al., 1986). The characterized antigens may not be protective because T cells may recognize different antigens (or epitopes) than do antibodies. An understanding of the immune system component that is responsive to coccidian infection is fundamental. We may be able to select specific antigens from specific stages of the parasite to stimulate that specific component of the immune response to protect the host. We can then employ means such as biochemical synthesis or recombinant DNA technology to produce protective antigen as a vaccine.

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